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ORGANIC NUTRIENT FACTORS EFFECTING ALGAL GROWTHS

Nicholas L. Clesceri, et al

Rensselaer Polytechnic Institute

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Absorbancies and regression analyses within algal exponential growth phases demonstrated the control growth rate for Selenastrum was 0.43 and for Anabaena was 0.34. Selenastrum growth rates were monitored using Lake George water as the diluent for the media employed. An inhibition in growth occurred. Halving the nitrogen concentration in modified Gorham's had no significant effect on growth rate.
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ORGANIC NUTRIENT FACTORS EFFECTING ALGAL GROWTHS

By

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Project 16010 DHN
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ABSTRACT

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SECTION I

CONCLUSIONS

The work undertaken and described has proven that increased algal growth has been exhibited upon addition of organic wastewater fractions to cultures of selected algal species. Based upon these data, it is expected that such effects may also occur in the natural regime. Several other conclusions resulted from this study.

1. The organic constituents of secondary effluents are in the main of low molecular weight.

2. The removal of nitrogen and phosphorus from treatment facility effluents may not completely obviate the possibility of algal proliferation in the receiving watercourse.

3. The siting of pollution control facilities and the treatment schemes to be provided should be carefully investigated, especially in those areas where minimal dilution may be expected.

4. Increased removal of organic carbon should be mandated in facilities proposed to discharge to low flow streams and essentially stagnant bodies of water.

5. An inhibition in the growth of *Selenastrum capricornutum* occurred when Lake George water (soft water oligotrophic lake) was used as the diluent for the growth media.

6. Nitrogen concentration could be lowered in modified tenth Gorham's medium without altering the growth rate of *Selenastrum capricornutum*.

SECTION II

RECOMMENDATIONS

Based on analysis of the results and experience gained, the following recommendations are made:

1. Further investigation should be undertaken to assess fully whether or not the Batavia effluent is unique in its growth rate enhancement potential.

2. It should be determined if other forms of treatment, eg, physico-chemical, produce effluents with such growth enhancement potentials.

3. The gel chromatography technique should be further investigated to determine whether such a tool may produce wastewater characterizations with applicability to treatment plant operation and effectiveness.

4. Further studies should be undertaken to ascertain the exact nature of those factors, within the fractions, responsible for the growth rate response noted.

5. Further studies involving the ascertainment of the effects of wastewater fractions on ultimate growth should be undertaken.

6. Carbon 14 uptake studies should be included in any further undertakings. The use of such technique will allow for an assessment of effects on photosynthetic activity.

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SECTION III

INTRODUCTION, OBJECTIVES AND TECHNICAL REVIEW

For many years man has been aware of the natural process of aging taking place in the earth's lakes. This natural process in the limit leads to degradation of the resource and a concomitant unfitness for use. This awareness has today become acutely heightened as a result of the acceleration of this natural aging process through the activities of man.

With the onset of the Industrial Revolution, man in his quest for progress and power began to destroy the environment, its land, air and water resources. Those activities which accompany civilization and progress, namely increased and unchecked waste discharge, increased utilization of land and fertilizers to meet an ever-expanding requirement for food and the rerouting and damming of streams have led specifically to a rapid acceleration of the destruction of the water resource (66, 85, 89, 90).

Eutrophication has become the shibboleth of the day. Skulberg's definition is adopted here as exemplifying the true interpretation; "the process of eutropication is primarily caused by the supply of plant nutrients to the aquatic environment even though other factors are involved, such as morphometric relations and the degree of biological utilization of the nutrient substances. The manifestation of eutrophication is the increasing of primary productivity; in extreme cases, the water bloom phenomenon developes and in stagnant water oxygen supply becomes depleted" (85).

The investigation of the effects of man-made wastes, perhapes the primal cause of the eutrophic phenomenon has received minimal examination to date and thus requires specific and intensive effort. The complexities of such investigations are compounded by the very nature of discharged wastes; they "contain everything" in the realm of nutrients (52).

The nutrient constituents of the aquatic environment responsible for these eutrophic phenomena, the main categories of interest being the macronutrients, nitrogen

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and phosphorus, the trace element micronutrients vanadium, molybdenium, etc and in several cases the accessory growth factors cyanocobalamin, biotin and thiamine, have been the subject of studies which have led to the development of perceptible results in some cases, and in other, foundations for speculation and hypotheses requiring further investigative scrutiny (77, 91).

The endeavors undertaken and documented in this investigation involve a hitherto neglected but specific aspect of algal metabolism, namely, the effect of wastewater organic compounds on the growth of algae. To attain this end, the organic component of the effluent from a modern well-operated conventional activated sludge system was separated into fractions according to molecular size by gel permeation chromatography. The fractions were then examined as to their possible effect on algal growth by standard culture comparison techniques. The results of the chromatography and the biological investigations are presented in the following chapters.

An examination and comparison of the effectiveness of thin film evaporation and freeze-drying for concentrating organics from natural and wastewaters was also determined (Appendix A). Both methods were studied as to their relative polymerization or hydrolysis effects and their recovery efficiency so far as organic carbon was concerned. In addition, the soluble organic component in municipal wastewater has been characterized and the effect of chemical-physical treatment has been shown (Appendix B).

Nutrient Factors in Algal Growth

1. The Natural and Synthetic Environment

No question has been raised as to the efficacy of nutrients for sustaining and being requisite for the growth of algae. The only query yet to be answered fully is what concentrations of specific nutrients are required for sustained growth of a nuisance algal bloom in a particular situation.

The investigations into the limiting requirements of nutrient factors have been carried on extensively in laboratory experiments; the use of which has at times been assailed as an unnatural system with no possible validity for extrapolation to the natural realm. However, Provasoli has stated the case for laboratory cultures rather succinctly, "It is often said that laboratory conditions are wholly artificial and that laboratory experiments at best vaguely approximate natural conditions. But the ecological arena is populated by the products of the continuous challenge of nature to the potentialities of the organisms... In fact, the challenges provided by nature are as strenuous to the organisms as are those inflicted on them by extreme conditions in exploratory laboratory experiments" (77).

The obvious analogy that an *in vitro* requirement or its physiological equivalent should of necessity be found *in vivo* has been borne out in extensive investigations. The requirements for all nutrient species save for nitrogen and phosphorus have been noted as being approximately the same *in vitro* and *in vivo*. The needs for nitrogen and phosphorus though are 10 to 100 times greater *in vitro*. If one realizes that the bloom may be concentrated by wind and density differences in the resource the algae may well have derived their required nutrients from an extremely large volume of water thus negating the need for the existence of high concentrations of these elements in the natural regime.

2. Eutrophication Case Studies

That the production of eutrophic conditions in the aquatic environment has been seriously accelerated by the discharge of waste is without question. Several monographs relating the changes for the worse caused by unchecked discharges document the definite implication of waste discharges as the primal causative factor in accelerated eutrophication (18, 23, 35, 68, 89).

Among the products of the eutrophic phenomenon are (1) dense mats of algae which render the water resource aesthetically unattractive to those seeking recreational opportunities, (2) loss of the economic value of lake shore property, (3) clogging of water treatment filters, (4) the production of odors and tastes, (5) discoloration of the water, and in the limit, (6) anoxic conditions and their coincident problems.

Since Hasler's fine description of the problem in 1947 (44), the literature has been replete with the examples of man's enhancement of the degradation of the aquatic environment. Lakes Washington, Green, Zoar, Tahoe, Clear, and even the Great Lakes, have been examined and found to be suffering from the general malaise symptomatic of eutrophication (7, 25, 42, 89). Neither has the estuarine environment escaped notice (83). The list of victims does not diminish as more notice is taken of the problem.

3. Macronutrient Requirements

As the body of information has grown regarding the eutrophic phenomenon, the causative agents have begun to receive scrutiny. Nitrogen and phosphorus have been implicated as the key limiting factor in the growth of algae in the natural environment (2, 24, 39, 48, 49, 50, 52, 56, 66, 77, 82, 89). This conclusion is logical if one considers the important part these elements play in bioenergetics and the synthesis of protein and their minimal levels of concentration in the environment. It should be noted that the preponderant attention focused on these elements has in the main resulted from the knowledge that their removal from discharges could be effected and that, for example, the environmental carbon concentration would never be completely subject to human control.

Phosphorus requirements. Although phosphorus, as noted previously, is an important macronutrient and has been regarded as perhaps the key element in the nutritional pattern of algae, contradictions developed by several investigators and the variability of results as to the exact nature of the limiting concentration are evident.

Tucker in his study of Michigan lakes found that the minimal concentrations of phosphorus available were not limiting to the growth of algae (91). Gerloff and Skoog (39) and Goldman (42) in their studies of eutrophic lakes found that phosphorus was not the limiting nutrient. The concentrations of phosphorus claimed to be limiting have exhibited striking disparity. Ketchum (48) indicates that in his experiments with Nitzschia closterium, a phosphorus concentration of 17 ug/l was limiting to cell division and the rate of assimilation of phosphorus. Rhode (80) has developed much information on the required concentration of phosphorus. He discovered that 0.2 mg/l of phosphorus as phosphorus was limiting for *Ankistrodesmus falcatus* and that the growth of *Scenedesmus quadricauda* increased as the phosphorus concentration was raised to 1.0 mg/l. However, he found that the addition of only 2 ug/l of phosphorus to "Bacteria filtered" Lake Erken water containing 10 ug/l of phosphorus produced full growth of *Asterionella formosa* while artificial medium containing 9 to 18 ug/l allowed for no growth of the organism. Based on these anomalous data, Rhode proposed the existence of a phosphorus sparing factor in the natural regime; that is one of several factors which principally change the phosphate dependence of the organism and thus facilitate its use of and coincident proliferation with low phosphorus concentration. Sawyer (82), as a result of his work on Wisconsin Lakes, has posited a concentration of 0.015 mg/l as a minimal requirement for the occurrence of nuisance blooms, since this concentration occurring at the time of the Spring turnover did not allow for the development of nuisance blooms and thus was indicative of a well-behaved lake. The data of Shapiro and Ribiero (83) led them to conclude that in the estuarine environment they studied, phosphorus was definitely not the limiting factor for the growth of blue-green algae in their experiments.

Also worthy of note are those instances of inhibition of algal growth by phosphorus. Chu (12) found phosphorus concentrations in excess of 8.9 to 17.8 mg/l inhibitory to the growth of selected algae. Rhode (80) in his investigations with *Asterionella formosa* found that, although 0.002 mg/l added to filtered Lake Erken water produced maximum growth, an increase to a level of 0.05 mg/l was inhibitory. He also ascertained that a concentration of 5 ug/l was inhibitory to *Dinobryon* and *Uroglena*.

Thus one can see that the phosphorus situation is a mosaic of alternating limitation, non-limitation, variability of limiting concentrations and finally inhibition. The results lead to the conclusion that phosphorus in many instances is limiting, the concentration range is low and narrow and organism dependent, and that the environmental requirements seem to be orders of magnitude lower than those resulting from culture experiments. The phosphorus sparing factor of Rhode noted previously has been posited as one explanation of this phenomenon and luxury uptake as another.

Nitrogen requirements. Nitrogen, as is the case with phosphorus, has been alternately implicated and acquitted in the specific investigations for a limiting factor in the growth of algae. The literature is replete with the results of investigations on the limiting nature of nitrogen (2, 24, 36, 38, 39, 49).

Gerloff and Skoog (39) found that nitrogen was the "primary limiting factor" for the growth of Microcystis aeruginosa in several Wisconsin lakes. Kratz and Myers (49) concluded from their data that nitrogen metabolism was the process determining the maximum growth rate for the algal species studied, Anabaena variabilis, Anabaena cylindrica, Anacystis nidulans, and Nostoc muscorum. Dugdale and Goering (24) discovered in their study of diatom blooms in the Sargasso Sea that nitrogen was the key limiting factor, while on the other hand, Goldman found little evidence for a nitrogen or even a phosphorus limitation in his studies on Clear Lake (42). Also Shapiro and Ribiero (83) on the Potomac Estuary found nitrogen to be non-limiting to the growth of blue-green algae, a function they infer of the nitrogen fixation capability of such algae. But such a conclusion may not be extrapolated to all blue-green algae as Fogg (33) has noted the ability of fixation has been discovered in only 21 species belonging to 8 genera. Even though the capability exists, it has been shown that atmospheric nitrogen is not generally as efficient as ammonia or nitrate in satisfying the metabolic requirements for nitrogen in blue-green algae. Kratz and Myers (49) showed that nitrogen fixation alone would only support 75% of the growth of Nostoc muscorum produced when nitrate was provided and used as the source of nitrogen.

As with phosphorus, investigations have led to the conclusion that nitrogen in certain concentrations can be inhibitory to the growth of selected algae. Ketchum in his excellent review article (48) has noted that Zobell found the upper limits of nitrogen concentrations for the growth of Nitzschia closterium to be 0.7 mg/l (NH_3), 70 mg/l (NO_2) and 560 mg/l (NO_3), all as nitrogen. In contrast to these data, Chu (12) found concentrations of nitrogen in the form of an ammonium salt in excess of 5.3 mg/l as nitrogen to be inhibitory to Staurastrum paradoxum; and 17 mg/l N when a nitrate source is used to be inhibitory to Asterionella gracillima.

The limiting concentration of nitrogen thus varies from species to species within a wide range of concentrations. Again we are presented with a mosaic of alternating limitation, non-limitation and inhibition dependent on species and resource investigated.

4. Micronutrient Requirements

Inorganic micronutrients. The elements required by algae for proper growth and development are many. Eyster has noted that 20 elements may be required in the biochemistry of green plants and has presented them in tabular form (26). The data are reproduced herewith as Table 1.

As can be seen from the tabulation, the sole difference between macro and micro nutrients rests on the relative concentrations of each set required for sustained growth of algae.

Eyster has noted that the trace elements are commonly employed as metal constituents of enzyme systems which enter into biological reactions (26). Provasoli (77) has noted in his review that molybdenum, copper, manganese, vanadium and cobalt were demonstrated to be essential to the growth of Chlorella, Scenedesmus and Anabaena and infers from such that the requirements for these elements hold for all species of algae. The need of nitrogen fixation for trace elements, such as boron, calcium and molybdenum has been pointed out by Eyster (26).

Since the quantitative requirements of algae for these trace elements are minimal the in vitro determination of limiting concentrations is fraught with difficulty. Two major problems may cast a cloud on the attempts at the determination of limiting concentrations. The lack of purity of the nutrient salts used in medium makeup thus leading to trace element contamination, and the seeming dependency of trace element requirements on medium composition (92). Confusion as to the limiting concentration

Table 1. Elements Required by Green Plants

Macronutrients (10^{-2} - 10^{-4} M)	Micronutrients (10^{-5} M and Less)
C, H, O, N	Fe, Mn, Cu, Zn
P, S, K, Mg	Mo, V, B
Ca*	Cl, Co, Si
Na**	

* Except for algae where it is a micronutrient
** For blue-green algae

may also develop as a result of the similarity of symptoms produced by a lack of several elements. Notwithstanding the above, the requirements for trace elements are absolute but the limiting concentrations of the elements have not been definitely fixed. It is hoped that the implementation of new purifying techniques in the production of the nutrient salts will have a salutory effect on attempts at the determination of a quantitative requirement.

Organic micronutrients. Noteworthy compounds also required in microquantities for the growth of some algal species are the organic growth factors, vitamin B_{12}, thiamine and biotin (22, 32, 77). The interest in the presence of algal forms of a requirement for these items developed out of the realization that the early cultivation of algae in the laboratory relied on the placement of soil extracts of unknown organic composition in the medium. Provasoli considers the pattern of vitamin needs stereotyped as only the above factors either singly or in combination seem to be required by algae. Droop has prepared a tabulation of the percentages of strains in each division requiring these factors (22). Reproduced in Table 2 are the data of Droop.

Vitamin B_{12} is required by more species than either thiamine or biotin (77). This contention is borne out by Droop (22) who posits that the species requiring the growth factors, 80% require vitamin B_{12}. Provasoli has shown that the sensitivity, depending on the organism involved, is 0.1 ng/l to 5 ng/l of vitamin B_{12} (77). Sensitivity, having only empirical value, is defined as the smallest amount of the vitamin giving measurably increased growth as compared with a control.

The determination of the concentration of vitamins required in the test medium or environment is complicated by the low levels and in the case of B_{12} by the fact that many species are able to employ analogues of B_{12} and more specifically several blue-green species and Chlorella have been found to be able to synthesize B_{12} in a deficient medium (22, 50, 56, 77). Kratz and Myers state that numerous attempts to elicit a growth response from selected blue-green algae, Anabaena variabilis, Anacystis nidulans and Nostoc muscorum, by the addition of vitamins to a culture medium have proved fruitless (49). Droop (21) reviewing the

Table 2. Vitamin Requirements of Algae as Reported in the Literature, After Droop (22).

				Strains Requiring				
Division	Total	Auxotrophs	Non-Auxotrophs	Only B_{12}	Only Thiamine	B_{12} and Thiamine	Thiamine and Biotin	B_{12} Thiamine and Biotin
Chlorophyta	47	22	25	10	7	5	0	0
Euglenophyta	10	10	0	0	1	9	0	0
Chryptophyta	11	11	0	2	1	8	0	0
Pyrrophyta	17	17	0	12	0	0	1	4
Chrysophyta	13	12	1	2	1	6	1	2
Bacillariophyta	54	21	33	11	6	4	0	0
Phaeophyta	1	0	1	0	0	0	0	0
Rhodophyta	1	1	0	1	0	0	0	0
Cyanophyta	25	1	24	1	0	0	0	0
Totals	179	95	84	39	16	32	2	6

sensitivity of data for various species and the lowest level of B_{12} detected in the sea (0.1 ng/l) proceeds to allow as that such a concentration could afford a crop of 25 million Skeletenoma, equal to the highest number ever recorded in nature. From this he supposes that B_{12} is always in excess in the sea. Daisley, however, disputes this contention as he feels that the growth rate and not yield is the more important measurement as a certain rate of growth is required to overcome losses due to predation and sinking (16).

5. Growth Substances

For the last thirty years, literature has been produced regarding the effect of organic growth substances on the growth of algae. Growth substances being differentiable from the growth factors in that the latter may be considered essential to the growth of certain species, that is, be an absolute requirement; while the former produce increased growth rates and yield but their absence from an otherwise full complement medium does not inhibit growth.

The major studies to date have involved the determination of possible stimulatory effects by auxins, plant growth hormones of non-specific nutritional value. The first report of the effect of these compounds on algae was produced in 1937 by Yin (95). He found that the addition of 10 mg/l of 3-indoleacetic acid (C_{10} H_9 O_2N) produced a 20% increase in the mean diameter of Chlorella vulgaris but did not measurably increase the growth rate. At the same time Leonian and Lilly (55) reporting on their studies of the effects of heteroauxin (3-indoleacetic acid) on Chlorella pyrenoidosa, C. minata, Cystococcus cohaerans, Oocystis naegelii and Scenedesmus flavescens stated that insofar as the species they investigated were concerned heteroauxin was inhibitory to growth rather than stimulatory. In 1938 Pratt (73) in studying auxin effects on Chlorella vulgaris produced evidence to show that such compounds stimulated the rate of multiplication of the organism. In his experiments with 3-indoleacetic acid, 3-indolebutyric acid and 3-indolepropionic acid he was able to show that concentrations of 50 mg/l of each of the compounds placed individually in the medium greatly increased cell multiplication and that lesser concentrations in the order of 10 mg/l produced smaller but very clear increases in the rate of multiplication of the organisms. The effectiveness of the three compounds was, however, not the same. 3-indoleacetic acid gave the greatest increase, almost twentyfold, while the 3-indolebutyric acid produced the least increase. Pratt was unable to detect any change in the mean diameter of the cells. Coincident with these experiments he also tested the effect of various compounds, among them potassium acetate, glucose, acetic acid, allanotoin, and glycine, in the same concentration, based on carbon content, as would be added to the medium by 50 mg/l of 3-indoleacetic acid. These compounds produced an increase in cell multiplication, with the greatest increase caused by the addition of glucose; however, the increases in no way equaled those demonstrated after the addition of the 3-indoleacetic acid. Brannon and Bartsch (9) also investigated the effects of auxins on algal growth. Based upon their experimental work with *Chlorella vulgaris* they found that auxins had a salutary effect on the 12 day yield of the test organism. The increase in cell number over the control ranged from a low of 73.1% produced with a concentration 6.7 ppm of indolepropionic acid to 261.4% with phenylacetic acid in a concentration of 33.3 ppm. Intermediate increases were noted for other auxins, eg, 119.0% increase for 10.0 ppm of 3-indoleacetic acid, 166.5% for 6.7 ppm of indolebutyric acid and 172.4% for 33.3 ppm of naphthalenacetic acid. They also looked into the effect of such compounds on *Coccomyxa simplex* and *Mesotaenium caldariorum*. The data regarding these species are minimal but it is stated in the discussion portion of their paper that "suitable concentrations of naphthalenacetic, indoleacetic, indolebutyric, indolepropionic and phenylacetic acids applied in sugarfree inorganic culture media stimulated reproduction in *Chlorella vulgaris*, and *Coccomyxa simplex*, but not in *Mesotaenium caldariorum*." It should be noted for further reference that the substances tested were dissolved in ethanol before addition to the culture media. Brannon and Sell (10) in 1945 found that concentrations of 10 ppm and 20 ppm of 3-indoleacetic acid stimulated the growth of *Chlorella pyrenoidosa* and produced greater than a fourfold increase in dry weight after thirty days. Meanwhile Manon (58) discovered that a concentration of 10 ppm of 3-indoleacetic acid was inhibitory to the growth of *Chlorella vulgaris* and that a concentration of 50 ppm was definitely toxic. He attributed the disparity between his results and those of Brannon and Bartsch (9) to the possible use of different strains of the organism, due to the poor taxonomical situation with *Chlorella* existing at that time, and to possible differences arising out of a variation in the purity of the acid used in the two experimental procedures.

Conrad and Saltman (15) in their treatise on growth substances note that Sven Algeus in 1946 performed extensive experimental work on the effects of 3-indoleacetic acid, 3-indolebutyric acid, 3-indolepropionic acid, naphthalenacetic acid and phenylacetic acid on cultures of *Chlorella vulgaris* and several species of *Scenedesmus*. It is reported that Algeus found that a concentration of 0.1 mg/l of 3-indoleacetic acid exhibited a threefold increase in cell number for *Scenedesmus obliquus* and that the other compounds produced measurable but not as profound effects. Further tests with the 3-indoleacetic acid and cultures of *S. acuminatus*, *S. dimorphus*, and *S. quadricauda* either produced no increase or inhibited growth. In his work with *Chlorella vulgaris*, Algeus found that one strain of the species responded with a threefold increase in cell number at a concentration of 1.0 mg/l of 3-indoleacetic acid, while the other strain evidenced the effects of several inhibition at this concentration. The foregoing data lend credence to the first contention of Manos cited above.

Williams in 1949 (93) found that the use of 3-indoleacetic acid produced an increase in the wet weight of disks cut from the brown alga *Laminaria agardhii*; but the article reporting his work shows no replicate data and it is noted therein that almost half of the treated samples disintegrated at concentrations ranging from 0.1 mg/l to 10 mg/l. Jacobs (46) reported that 3-indoleacetic acid in a concentration of 100 mg/l had a stimulatory effect on rhizoid formation in *Bryopsis plumosa*. He found that no rhizoids were formed in the controls. Davidson (17) shortly thereafter reported that fronds of *Ascophyllum nodosum* and *Fucus evanescens* responded to concentrations of 3-indoleacetic acid from 10^{-9} M (175 ng/l) to 10^{-4} M (17.5 mg/l) with 10^{-5} M (1.75 mg/l) giving a 57.1% increase in apical growth. A 10^{-7} M (0.0186 mg/l) concentration of naphthalenacetic acid produced this maximum growth, but concentrations in excess of 10^{-6} M (0.186 mg/l) proved to be inhibitory. Corresponding concentrations of 3-indolebutyric acid also proved inhibitory. In the case of Fucus evanescens growth was promoted by 3-indoleacetic acid in concentrations from 10^{-8} to 10^{-4} M and by 10^{-8} M concentrations of naphthalenacetic acid, and 3-indolebutyric acid. It should be noted that, as with the earlier work on the effect of auxins, the auxins used here were dissolved in an alcohol solution.

Provasoli (75, 76), studying the effect of auxins on the green alga Ulva lactua, concluded that the initiation of germlings and elongation of the filaments were brought about by the combination of kinetin and 3-indoleacetic acid in concentrations of 0.10 mg/l and 0.05 mg/l respectively.

The effect of auxins on the growth of diatoms has been minimally investigated. Bentley (8), in experiments with the marine diatom Skeletonema costatum, found that 3-indoleacetic acid affected chain length and cell multiplication. Skeletonema under natural conditions in the sea exists as a long chain organism with the chains very often containing 50 or more cells. In a synthetic medium, it was discovered that the organism was essentially unicellular with a few chains of 2 to 3 cells evident. Optimum concentrations of 3-indoleacetic acid, 10 ng/l to 100 ng/l resulted in an improvement of chain length, an average of 8 cells per chain after treatment, and a doubling of cell number.

Miller and Fogg (65) in their study of Monodus subterraneous elicited evidence that 3-indoleacetic acid had no effect whatsoever on the growth of the organism. In their wide-ranging study they also noted that several organic substrates, including glucose, tryptone and the growth factors thiamine and vitamin B_{12} also had no effect on the growth of the organism; however, it was found that several of the substrates tested were able to serve as sources of either phosphorus, eg, sodium glycerophosphate, or nitrogen, eg, glutamine, urea and succinamide.

In 1958, Bach and Fellig (6) and Street et al (87) reported on their findings that ethanol led to a stimulation of the growth of Chlorella vulgaris. Street and his colleagues discovered that the addition of an ethanolic solution of 3-indoleacetic acid produced a 160% increase in cell number, while an aqueous solution of the same compound in the same concentration only allowed for an 11% increase. Bach and

Fellig (6) at the same time reported that while small concentrations of ethanol in the culture medium led to a twofold increase in cell number, when the same experiments were conducted on a shaking table the stimulatory effect disappeared. This result led them to postulate that carbon was limiting in the earlier experiments on auxin effects and that the agitation of the cultures would have, in removing the limitation, negated the requirement for carbon and thus reduced measurably the stimulatory auxin effects noted. Their further experiments with auxins bear out this contention as they were unable to find any stimulatory effect. Although the above work casts doubt on the validity of the conclusions drawn from the early work of Brannon et al, the fact remains that the work of Algeus, Bentley and Conrad and Saltman (14), wherein the use of alcohol was scrupulously avoided and no carbon limitation was evident, has not been refuted.

Prakash and Rashid (72) in studying the effects of humic substances on algal growth found that humic acid fractions obtained by gel permeation chromotography exerted a stimulatory effect on the growth of Gonyaulax tamarensis. The effects noted were: increasing yield, growth rate and ^{14}C uptake. They attribute these effects at concentrations of 2 ug/l to 35 ug/l to the fact that these small amounts may act as stimulants and may be involved in cellular metabolic processes. They refer to other experimental work which may indicate that the humic substances act as sensitizing agents enhancing the permeability of the cell membrane and consequently, increasing the uptake of nutrients from the surrounding medium.

6. Carbon

Recently a controversy has developed over the true nature of the key limiting factor for algal reproduction in the natural environment. Kuentzel (51) has proposed that the key limiting factor is carbon. He reasons that carbon as CO_2 is the major nutrient, since nuisance blooms of algae are always associated with the discharge of excessive amounts of organic matter to the resource. He infers from these postulates that the organic matter acted upon by the bacteria produce the carbon necessary and that the large amounts of carbon required for the production of such blooms cannot be made available from the atmosphere or from HCO_3 dissolved in the water. He supposes a "perfect" symbiosis between the bacteria and the blooming algae and states that before a bloom of algae may occur a bloom of bacteria must have taken place.

The fact that carbon is a major nutrient required by the algae and that the unchecked discharge of carbonaceous material could be the source of such a carbon requirement is not in dispute. What has been seriously questioned is his contention that the concentration of phosphorus in the aquatic environment is already sufficient to support massive blooms of algae and that any steps taken to require the reduction of the amount of phosphorus discharged to the environment without at the same time taking out more of the carbonaceous material would be self-defeating. Shapiro (84) and Kuentzel (54) have exchanged statements regarding their personal and professional interpretations of the facts relating to phosphorus concentrations in the ecosystem and their suitability for algal growth. Shapiro has stated that the phosphorus requirements of the growing algal cell are essential and well known and that a concentration in the environment suitable to provide such amounts is a sine qua non. The fact that low concentrations are always found in the water during a bloom he attributes to previous phosphorus uptake by the algae. Kuentzel on the other hand argues that algae can produce numbers in excess of the stoichiometrically determined requirements for the cells and that only a minimum amount of phosphorus is required for a bloom. This amount already being present, he feels that the removal of phosphorus from discharges is a wasted effort. What must be taken into account, however, is the rapid turnover of phosphorus in the aquatic environment, a matter of 3 to 5 minutes according to Rigler (79), the ability of algal species to utilize organic phosphorus (12, 80) and the species dependency of the required phosphorus concentration in the environment. It would seem from the data previously presented that perhaps in certain situations the attainment of phosphorus concentrations in the natural realm allowing for no algal proliferation might well be impossible.

Saunders in his article on the interrelationship between phytoplankton and dissolved organic matter (81) has noted that the activity of the organic matter, although comprising only a very minor portion of the total number of factors affecting algae, may be very significant when integrated into the total picture. He ascribes to organic matter four possible functions relating to algal growth: (a) utilization as an energy source or provision of an essential nutrient required in the building of cellular material, (b) action as an accessory growth factor vitally essential to survival of the species, (c) toxicity leading to inhibition or complete cessation of growth and finally (d) the organic compounds may act as a chalating agent forming complexes with trace metals in the environment and thus produce beneficial or detrimental results depending on the circumstances involved.

Since in the process of evolutionary development, algal forms have arisen which cover the range from obligate autotrophy to auxotrophy and finally, in some species under certain conditions heterotrophy with all the shadings in between evident, it would not be amiss to prepose that some of the organic compounds extant in a wastewater effluent could affect the growth of algae in the natural environment. As a specific example, one can note the occurrence in treated wastewater effluents of vitamin B_{12} previously noted as a required accessory growth factor for many algal species.

Concentration and Separation

1. Freeze-drying

The use of the freeze-drying technique to preserve and concentrate biological samples has been in use for at least fifty years. But it was the requirements for plasma and dried penicillin during World War II which brought the technique to fruition.

Freeze-drying, simply stated, is the removal of water from a frozen sample via sublimation in vacuo. The beauty of the method as a means of preservation is related to the fact that the material is locked into the ice matrix during prefreezing and remains in position during the dehydration, thus affording no opportunity for interaction.

Theory. The major portion of the following discussion is based on two articles by Merryman which in combination present an excellent presentation of the factors involved in the freeze-drying technique (61, 62).

As pointed out by Merryman, the freeze-drying process involves the harmonious integration of three distinct factors: (a) the provision of heat to provide the energy required for sublimation of the ice, (b) the transfer of the water vapor sublimed through the specimen already dried, and (c) the removal of water vapor from the specimen surface.

Assuming that the water vapor is removed, the function of the introduced heat and its interrelationship with the sublimation procedure, can easily be explained. The sample after being frozen is composed of ice crystals rigidly confined in a crystal lattice with the solute locked within this matrix. The water molecules within the lattice are in constant violent motion and it is statistically probable that a molecule may through its violent motion overcome the energy barrier and escape from the ice crystal. If heat is provided to the system, most commonly by conduction, the probability of escape is increased. When the water molecule escapes to the more unrestricted vapor state it takes with it the latent heat of sublimation as energy. At -25° to -30°C Greaves has calculated that this is 672 calories/gram (43).

Since the escaping molecule has removed with it an amount of energy equal to the heat of sublimation, the temperature of the remaining ice mass will drop. If heat is added to the system an equilibrium will be set up so that in the end the energy removed by the subliming water molecules will just balance that amount added by the external heat source.

The foregoing has been presented on the basis that all water vapor and its coincident energy is removed from the system. Such is not, in practice, the true case.

As the ice sublimes, the atmosphere above the drying surface becomes suffused with water vapor. As this concentration of vapor increases, the probability of a return to the ice crystal becomes greater. If the return rate increases, the temperature of the sample will increase, positing a fixed heat input, as the returning molecule will bring with it the energy it originally removed. In the limit, the specimen will melt, thus the second and most important consideration is the prompt removal of the water vapor from the surface of the dried specimen.

The only force driving water vapor across the dried specimen is the concentration gradient. The vapor produced at the drying surface must diffuse through the already dried specimen to the specimen surface. To achieve this effect, the equipment in use has been designed to achieve almost zero vapor concentration at the specimen surface. For without this condition, the rate of diffusion through the dried specimen being low (a shell of dried material 1 mm thick reduces the drying rate by a factor of 1,000) the sample would eventually cease sublimating and melt. A zero vapor concentration at the specimen surface is achieved by having the vapor condense on a surface and thus be effectively removed from the system. To achieve this end the mean free path must be large, ideally equal to the distance between specimen surface and the condenser. This is the sole purpose of the vacuum system to enlarge the water vapor mean free path by removing almost all gas molecules from the area around the specimen surface and condenser and also from the interstices of the dried sample.

From the foregoing it can be seen that the controlling factor for freeze-drying is the rate of diffusion of the sublimed water vapor and the sole motive power for this transfer of water vapor from the drying boundary to the condenser surface is the concentration gradient.

2. Thin Film Evaporation

The idea of conducting evaporation under vacuum was first conceived by Howard, who invented a vacuum pan in 1812 (98). By using a jet condenser and an air pump he reached a boiling point of 60°C. The study of evaporation under artificially reduced pressure led to the development of multiple effect evaporators. The importance of moving the liquid itself relative to the heating surface was realized early in the Nineteenth Century. In 1830 Dubrufaut described an ideal evaporator for sugar liquors in the following words:

> "Concentrate the juice as rapidly as possible so as to avoid any alteration; give the liquid to be evaporated great speed, and, if possible, accelerate the movement of the liquor so as to evaporate in the form of a thin film."

The concept of thin film, however, took a practical shape only towards the close of the Nineteenth Century in the construction of the Lillie evaporator, which was patented in 1890 (98, 99). There have been a number of improvements since then in which this concept has been usefully employed. The laboratory scale rotary thin film evaporator is a development of the late forties. Partridge (100) has described such an apparatus for use in the concentration of small quantities of liquid at low temperatures under partial vacuum. The present-day rotary thin film evaporator is only a slight modification of the Partridge apparatus (evaporator). It is surprising to note that although this apparatus is used in almost every laboratory for concentrating small amounts of liquids, there is practically no literature evaluating its performance.

Theory. The process of thin film evaporation can be divided into four basic steps: 1) introduction of heat to supply the energy necessary for evaporation, 2) formation of thin film for efficient heat transfer, 3) evaporation of water from the thin film vacuum, and 4) removal of water vapor with the help of a water aspirator or a vacuum pump.

The process is similar to distillation. Applying the principle of differential distillation, Fredette (101) arrived at a simple mathematical relationship for calculating the recovery of organic materials dissolved in water. The relationship is expressed as:

$$\ln(n_o/n_1) = \overset{x_o}{x_1}\ dx/\ (cx-x) = (1/(c-1))\ (\ln(x_o/x_1))$$

where

n_o and n_1 are the initial and final moles of the solvent

x_o and x_1 are the initial and final mole fraction of the solute in the liquid.

c is a constant and its value is dependent on the vapor liquid equilibrium curve for the system. Fredette (101) assumed this curve to be a straight line while arriving at the above relationship. In this case c is the slope of the straight line.

The above relationship can be used to find the recovery of known compounds in the solvent. Fredette (101) obtained a very good correlation between the experimental and theoretical values based on the above relationship.

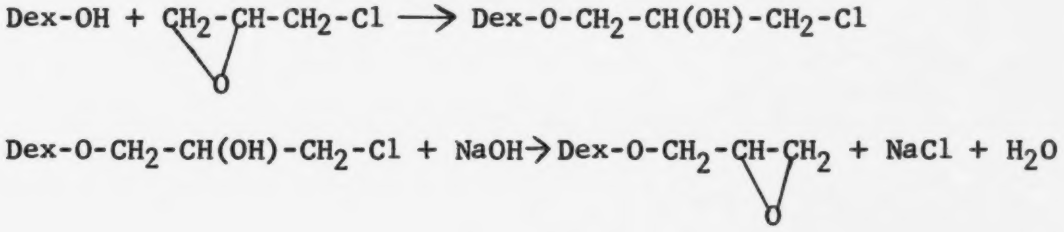
The investigations related to these concepts within this report are discussed in Appendix A.

3. Gel Permeation Chromatography

The separation of substances differing in molecular weight by passing a solution containing these substances through a bed of gel grains is termed gel permeation chromatography or more simply gel filtration. Many materials have served as a source of polymer gels. Among these are agar, starch, polyacrylamide and polyvinyl alcohol (53). The most widely used material has become dextran first described for use as a gel by Porath and Flodin in 1959 (70).

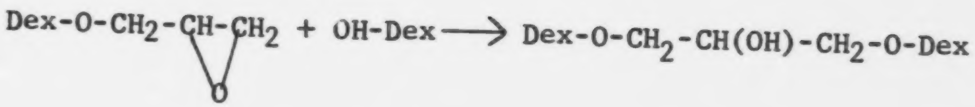
Sephadex dextran gels. Dextran is a high polymer carbohydrate produced during the growth of Leuconostoc mesenteroides on sucrose. The soluble polysaccharide developed consists exclusively of glucose and contains a preponderence ($> 90\%$) of α- 1,6 glycosidic linkages. Since it contains 3 hydroxyl groups per glucose unit, it is water soluble.

The reaction occurring upon the mixture of an alkaline dextran solution and epichlorhydrin proceeds exothermally to form polymer chains cross-linked with glyceryl ether bonds. The reaction described first by Flodin (31) in his dissertation and reproduced by Determann in his monograph (20) is as follows:



$$\text{Dex-OH} + \underbrace{\text{CH}_2\text{-CH}}_{\text{O}}\text{-CH}_2\text{-Cl} \longrightarrow \text{Dex-O-CH}_2\text{-CH(OH)-CH}_2\text{-Cl}$$

$$\text{Dex-O-CH}_2\text{-CH(OH)-CH}_2\text{-Cl} + \text{NaOH} \rightarrow \text{Dex-O-CH}_2\text{-}\underbrace{\text{CH-CH}_2}_{\text{O}} + \text{NaCl} + \text{H}_2\text{O}$$



$$\text{Dex-O-CH}_2\text{-}\overset{}{\underset{\diagdown O \diagup}{\text{CH-CH}_2}} + \text{OH-Dex} \longrightarrow \text{Dex-O-CH}_2\text{-CH(OH)-CH}_2\text{-O-Dex}$$

As can be seen from the above equations, the dextran polymers are cross-linked as a result of the reaction of epichlorhydrin with two hydroxyl groups on two different polymer chains. Gels of vastly different swelling properties may be produced depending on the concentration and molecular weight of the dextran polymer and the concentration of epichlorhydrin. This swelling property directly affects the size of the accessible regions within the gel structure and thus allows for the production of gels capable of separation over a wide range of molecular weights. The structure of a portion of the Sephadex dextran gel is as depicted in Figure 1.

From experimentation it has been found that the swelling properties of the gel increase with decreasing concentrations and molecular weight of dextran and decreasing concentrations of epichlorhydrin.

Theoretical aspects. Several numerical constants useful in the interpretation of gel filtration data have been advanced. These constants have been developed from experimentation with the gels in a columnar format. The setup is used almost exclusively in gel filtration work. The volumes existing in the gel column play an integral part in the determination of these parameters. The total volume of a gel bed (V_t) may be thought of as being composed of three separate volumetric entities:

$$V_t = V_m + V_i + V_o \qquad (1)$$

Where V_m is the volume of the bed taken up by the gel matrix, V_i is the inner volume, that volume taken up by the eluent inside the gel grains and V_o the void volume, that portion of the volume outside the gel grains.

The measurement of V_m and V_i are quite difficult but have been calculated by the manufacturer for the various Sephadex gels and may be used in the development of the column parameters.

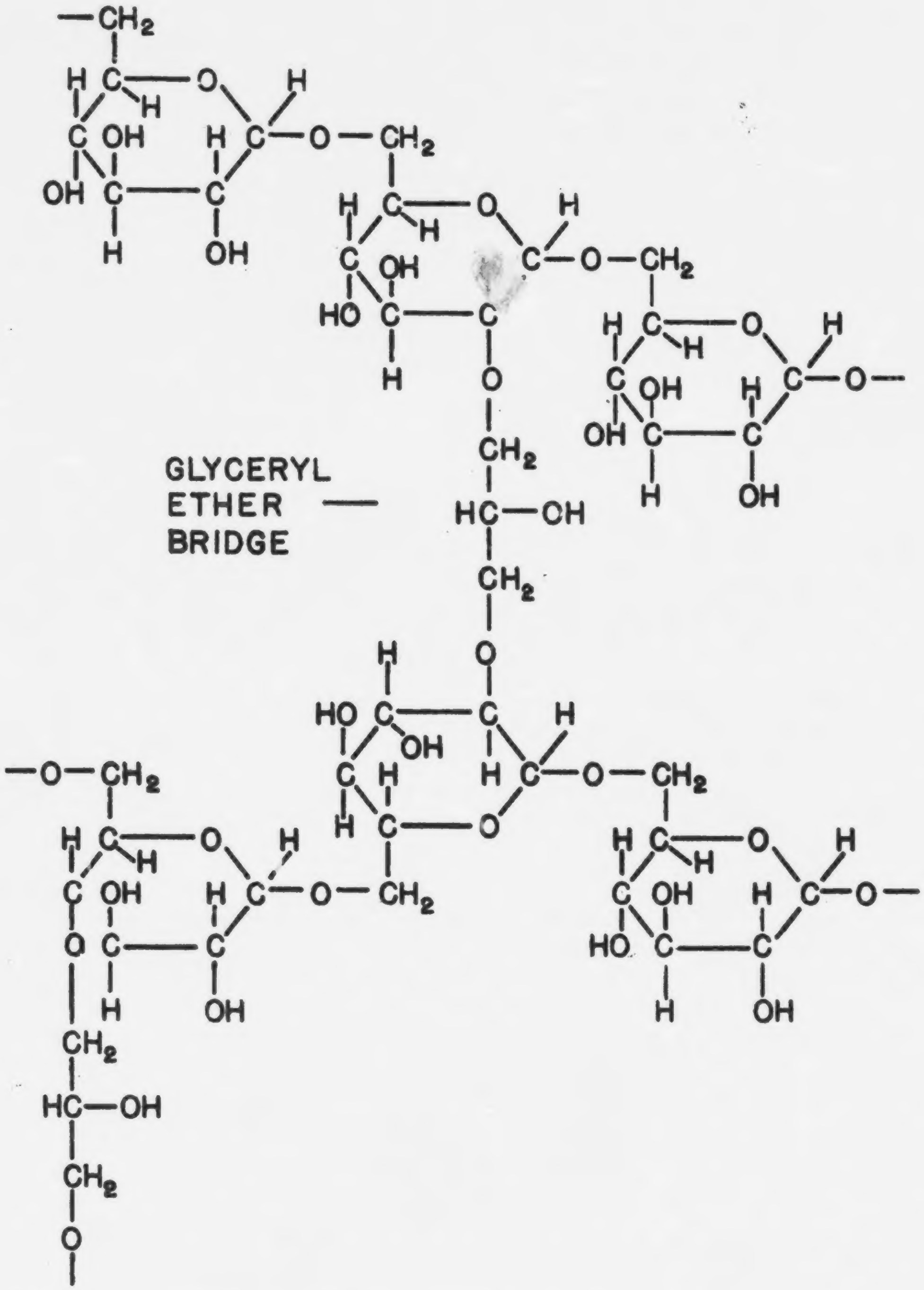

GLYCERYL
ETHER
BRIDGE

Fig. 1 Partial schematic structure of Sephadex dextran gel, after Determann.

Determann (20) has a presentation of the development of one such parameter. K_d the fraction of the inner volume available to the solute molecule was originally proposed by Wheaton and Baumann. The formula for this parameter is:

$$V_e = V_o + (K_d \times V_i) \qquad (2)$$

Since the elution volume (V_e) which is the volume of eluent passed through the column prior to the appearance of the molecule under study in highest concentration is a known factor the K_d may be calculated as follows:

$$K_d = \frac{V_e - V_o}{V_i} \qquad (3)$$

A compound completely excluded from the inner volume would have a K_d of 0 while a compound with a K_d of 1 would have the full inner volume of the gel available to it. Intermediate values of K_d would indicate compounds with only a portion of the inner gel volume available.

This parameter is independent of the geometry and packing density of the column and thus is useful for comparing work done with the same gel by different investigators. The sole drawback to the use of this constant resides in the uncertainty of the measurement of V_i.

To overcome this uncertainty, Laurent and Killander (53) developed the concept of a K_{av} constant. The difference between this constant and K_d rests in the fact that the total volume of the gel phase ($V_t - V_o = V_i + V_m$) instead of the inner volume (V_i) is used in the calculations. The elution volume of a substance may then be found by:

$$V_e = V_o + K_{av} \cdot (V_t - V_o) \qquad (4)$$

and K_{av} would be calculated from:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \qquad (5)$$

As with K_d this constant is independent of column geometry and packing density. Its main advantage is based on its calculation using clearly defined and easily obtained volumetric parameters.

Models for gel filtration. Gel chromatography having developed out of a few accidental observations was at first without a theoretical basis. Flodin in his investigations (30, 31) clearly outlined the prime factors essential to separations by gel chromatography: The elution volume of a molecule is independent of flow rate and sample concentration and is largely determined by its molecular weight. The analysis of experimental data collected over the past years has served to substantiate these contentions.

The previous discussion regarding the constants K_d and K_{av} is valid if one assumes that within the gel there are areas which are inaccessible to substances depending on their molecular size. Thus for each molecular size, there is a portion of the inner volume which is available by means of diffusion. An explanation of this exclusion principle has been advanced by Flodin (31). He reasons that there are regions near the point of cross-linkage in the gel where the degree of cross-linkage is so great that large molecules cannot diffuse into these areas. The number and extent of such areas are determined by the swelling properties of the gel and the size of the molecule under study. For the chromatographs produced during gel filtration to exhibit the degree of reproducibility that has been demonstrated, it would be required that the molecules have sufficient time available to them in the gel column to diffuse fully in their respective regions of accessibility. Experimental evidence collected to date has verified this contention as it has been found that the elution volume of a given substance on a gel bed is independent of the flow rate in the column up to the realizable maximum flow rate.

Several geometric models have been proffered as explanation of the operation of the exclusion principle. Porath (71) in developing the initial postulate made the basic assumption that the inner volume (V_i) was composed of cones of radius R. It is obvious if one posits a spherical molecule of radius r, that the molecule will penetrate the cone of inner volume to a degree determined by the ratio of the respective radii, r/R. Porath in comparing the volume of the cone available to the molecule (K_d x V_i) to the total volume of the cone obtained an expression for K_d,

$$K_d = k\left(1 - \frac{2r}{R}\right)^3 \qquad (6)$$

Employing proportionality factors for r and R he was able to arrive at the final format of:

$$K_d^{1/3} = k_1 - k_2 \times M^{1/2} \qquad (7)$$

where M represents molecular weight. This basic equation has been checked extensively against the experimental data and found to be valid in most cases.

Subsequently, Squires (86) assuming that the inner volume was composed of cones, cylinders and cracks developed the following experssion:

$$\left(\frac{V_e}{V_o}\right)^{1/3} = K_1 - K_2M^{1/3} \qquad (8)$$

The expression has been found to be valid for a number of proteins fractionated on Sephadex gels but has not represented the experimental data equally well in all cases.

Laurent and Killander (53) following the basic premise of earlier investigators that the position of a molecule in the elution chromatogram is a function of the volume available to it have proposed another model for the gel structure. They posit what they consider to be the "simplest model," by assuming that the dextran chains are infinitely long, straight rigid rods distributed at random throughout the gel. Using this model, they are then able to employ the formula of Ogston (67) to calculate the available volume for a spherical particle in such a system,

$$K_{av} = e^{-\pi L(r + R)^2} \qquad (9)$$

In the above expression L is the concentration of rods in the gel expressed as cm of rod per cm^3, r is the radius of the spherical particle and R the radius of the rod. From data provided in the literature and in their experiments they calculated the K_{av} using the void volume, total volume and elution volume for various proteins and dextran fractions. The experimental data showed that the model hypothesized is able to satisfactorily explain gel filtration on the basis of a steric exclusion of solute molecules from the gel phase.

Restricted diffusion. Ackers (1) has proposed a mechanism of gel filtration on the basis of a decreased rate of diffusion into the gel structure for large molecules. It is assumed in this explanation that the diffusion rate was restricted due to steric effects and friction in the gel pores. But this explanation encounters difficulty when one considers that the position at which a substance is eluted does not vary appreciably with the rate of eluent flow. This indicates that the exclusion principle and not restricted diffusion is the operative mechanism as the flow rate would have an effect on the elution position if restricted diffusion was the mechanism (20, 53).

Summary. It may be well to reiterate the salient points regarding gel filtration namely, (1) the elution volume of a substance is dependent on its molecular size, (2) the elution volume does not vary appreciably with the rate of eluent flow up to the technically realizable maximum, (3) the elution volume is not temperature or concentration dependent and (4) the elution volume is directly proportional to the log of the molecular weight of the substance under study (3, 4, 20). These findings based on the analysis of experimental data led to the conclusion that in the majority of cases the operating principle in gel filtration is based on the exclusion of a substance from the gel interior due to steric factors.

SECTION IV

PROCEDURES AND RESULTS

Physical and Chemical Investigations

1. Sampling, Preliminary Treatment and Concentration

The relative merits of a grab sample versus a composite sampling procedure for the collection of the effluent to be fractionated and subsequently used in the biological analyses were thoroughly investigated.

Due to the obvious lack of a clearcut advantage for a short term composite over a grab sample and the unfavorable economic and time demands required for a long term composite, it was decided that all samples of effluent would be grab samples. At all times samples of effluent were collected prior to chlorination.

It was realized that in a study of this nature, care must be taken to maintain the integrity of the sample under investigation. To accomplish this end the following procedural steps were devised and implemented.

Samples were collected and stored in previous acid-washed 18 liter polyethylene containers and returned immediately to the laboratory. The time of travel involved no more than 5 hours. Upon arrival an alinquot of the wastewater was taken for total organic carbon analysis using a Beckman carbonaceous analyzer. Immediately thereupon preliminary treatment was undertaken. In rare instances, the time of delivery to the laboratory did not allow for the immediate implementation of preliminary treatment. In these cases the wastewater sample was placed in a cold room maintained at a constant temperature of 4^{o}C until preliminary treatment was undertaken on the succeeding day.

The flow diagram shown in Figure 2 depicts the total treatment scheme proposed for use in the study. To render the effluent amenable for fractionation and subsequent biological analyses, some means had to be obtained to remove the particulate matter extant in the wastewater.

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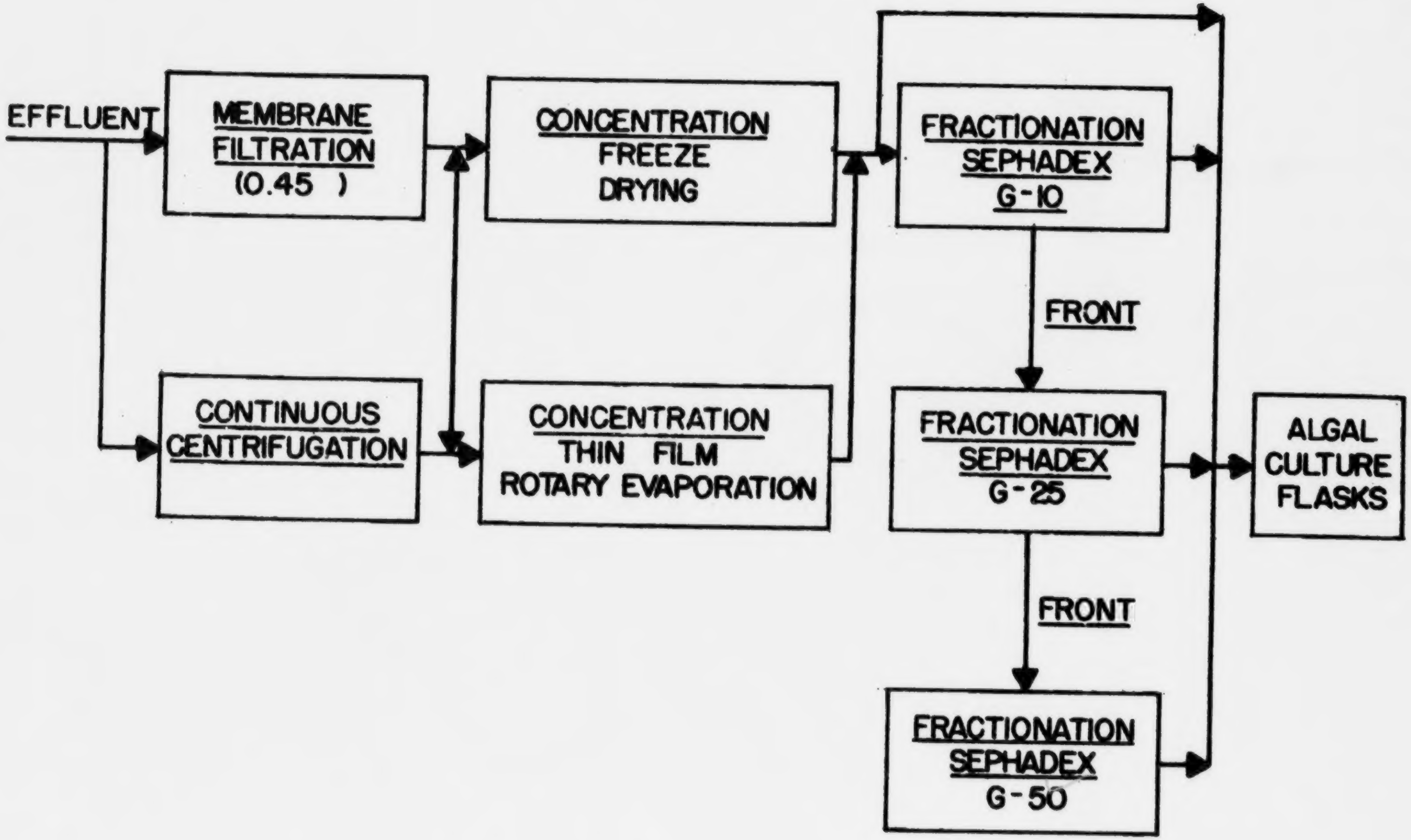

EFFLUENT
MEMBRANE FILTRATION (0.45)
CONTINUOUS CENTRIFUGATION
CONCENTRATION FREEZE DRYING
CONCENTRATION THIN FILM ROTARY EVAPORATION
FRACTIONATION SEPHADEX G-10
FRONT
FRACTIONATION SEPHADEX G-25
FRONT
FRACTIONATION SEPHADEX G-50
ALGAL CULTURE FLASKS

Fig. 2 EXPERIMENTAL PROCEDURE FLOW DIAGRAM

Continuous centrifugation. The first method investigated for the separation of particulates was continuous centrifugation. The equipment already available at the laboratory was a Lourdes continuous centrifuge model CFR-2. Several samples of wastewater were collected to assess the advisability of this method of preliminary preparation. It was found during the operation that the sample temperature after passage through the system increased appreciably to a point where it was higher than ambient. It was felt that such a temperature increase might have a deleterious effect on sample quality. This factor, coupled with the low rate of flow, average 5 l/hr, and the need for constant attention, induced a search for a more reliable and quicker means of separation.

Membrane filtration. In place of the above method a large membrane filter apparatus, 142 mm diameter, was employed and the sample filtered through a 0.45 u mean pore size membrane filter. The system was notably successful as it afforded ease and simplicity of operation, coupled with a high rate of separation.

The sample to be filtered was discharged into an 18 liter container that had been previously cleaned and acid-washed with hot dilute HCl (1:10 dilution). The container, equipped with an orifice at its base, was placed in an elevated position to provide sufficient head to fill the filter apparatus prior to the actual commencement of filtration. A water aspirator was used for vacuum filtration of the sample.

Subsequent to the filling of the collection flask and sooner if the level of particulates in the sample required it, the membrane filter was changed and the process repeated until all of the wastewater had been filtered. The contents of the collection flask were discharged into an acid-washed 18 liter container, which when filled was placed in the cold room. The minimum rate of filtration experienced during the investigation was 20 liters per hour. After the effluent sample had been filtered, an aliquot of the filtrate was removed for measurement of organic carbon.

After separation of the particulates ($>$ 0.45 u) from the soluble organic matter, a concentration step was employed. This step was taken to insure that the chromatographic column effluents would contain organic carbon in concentration readily detectable with the Beckman carbonaceous analyzer. It has been found that with a 2.5 x 100 cm column, the minimum sample organic carbon concentration that will allow detection of the column effluent fraction carbon concentration by the analyzer is approximately 100 mg/l.

An additional benefit is also enjoyed due to the use of a concentration procedure. If the sample organic concentration is increased, the number of passes through the gel column required to produce fraction volumes sufficient to be usable in the subsequent biological analyses are proportionately reduced.

Thin film rotary evaporation. At first thin film rotary evaporation procedures were attempted. The experimental apparatus included a Calab thin film rotary evaporator and a 500 ml cold finger condenser filled with ethylene glycol. The ethylene glycol was maintained at or below 10°C with the aid of a Forma cold finger.

This concentration procedure proved very troublesome. The apparatus, as constructed, required continuous monitoring to insure proper operation, especially the constant sample delivery accessory. When left unattended, the system was highly erratic and on one occasion an overnight concentration attempt resulted in complete filling of the apparatus, including the vacuum pump, with sample wastewater.

In addition to the operational difficulties, it was found that it was necessary to raise the temperature of the wastewater sample to approximately 34°C to insure reasonable evaporation rates. As with the continuous centrifugation step, it was felt that this elevated temperature would have a deleterious effect on the sample composition, and that, under the circumstances, another more reliable and less troublesome concentration procedure should be sought.

Freeze-drying. The final concentration procedure investigated was freeze-drying. A Virtis 10 liter large port freeze-dryer was obtained, and fitted during its manufacture with 16 3/4" inlet ports to allow a greater rate of sublimation. The apparatus is claimed by the manufacturer to be capable of concentrating 8 liters during a single run. However, it was found that for best operation 5 liters was the optimum load that could be imposed on the system at any one time. Thus, the maximum rate of concentration using the above system was 5 liters per day.

The freeze-drying procedure followed firstly involved the transfer of the filtered wastewater to ten 1200 ml freeze-drying flasks. A measured 500 ml volume of the filtered wastewater was placed into each freeze-drying flask. The sample flasks were then sequentially shell frozen by rapid manual spinning in a dry ice acetone bath (-61°C), and then placed on the 3/4" ports of the concentration apparatus. A McLeod gauge was integrated into the system to give an overall value for the vacuum attained during its operation. Using a high volume Welch Due-Seal vacuum pump, a vacuum of less than 10 microns mercury was maintained throughout the freeze-drying operation, except during the one minute equilibration period following the application of a flask to a freeze-dryer port. During this equilibration period a maximum vacuum reading of 100 microns mercury was noted. At no time were sample flasks placed on the apparatus less than one minute apart. This step was taken to insure that sudden overloads were not imposed on the system.

Initially, severe difficulty was encountered in the operation of the system. The flasks were geared to be used with either Pyrex glass connectors or stainless steel connectors, both items of thin wall construction. The initial samples were concentrated in flasks using glass connectors of thick Pyrex glass and with the Virtis filters in place in the flask caps. With this setup in use, a melt-back phenonmenon occurred, essentially ruining the concentration procedure being undertaken. Thereupon, it was decided that the use of the filters in the flask cap would be foregone, and a flask connector of larger inside diameter used to connect the flask to the freeze-dryer port. These two steps completely corrected all difficulties experienced with the system.

It is surmised that the melt-back phenomenon had been occurring due to constriction of the water vapor mean free path resulting from the use of a flask connector with a smaller inside diameter than recommended by the Virtis Company. Without the escape of the water vapor and its concomitant heat of sublimation, the temperature of the sample rose, leading eventually to melt-back.

Once the 500 ml samples had been reduced to approximately 50 to 75 ml, another 500 ml of the wastewater was added to each flask and the composite again shell frozen in the dry ice acetone bath and introduced onto the freeze-dryer. This sequence was repeated until the total volume of effluent collected, save for a minor amount retained for use as a flask wash, had been concentrated.

After the concentration had been completed the concentrate was filtered through a 0.45 micron membrane filter. The flasks were washed with small volumes of unconcentrated effluent and this wash also introduced onto the membrane filter. The filtrate obtained was stored in the cold room in a Pyrex reagent bottle. The solids remaining on the membrane filter was acidified with 0.1 N HCl and the resulting organic material also stored in a Pyrex container and placed in the cold room.

Upon completion of this step the organic carbon concentrations of the filtrate and the acid wash were measured. Combining these data with the known volumes of both portions, the total organic carbon content of the concentrate was calculated. Using this figure, and the organic carbon content of the original sample, a recovery factor was determined.

Criteria of selection. It was considered to be of prime importance that the effluent selected for fractionation and subsequent biological analysis be truly representative of secondarily treated wastewaters. To this end, two criteria were posited as guidelines for the selection of the wastewater sampling site. These criteria were: (a) low effluent organic carbon content, thus presupposing an efficiently operated wastewater treatment plant, and (b) a wastewater treatment facility utilizing a form of treatment applicable for use in presently planned and future large scale regional facilities.

Effluents investigated. In line with the above guidelines, three facilities were investigated as possible sources of effluent.

Eastern-Western wastewater treatment facility. The Eastern-Western facility located in Albany County, New York was the first plant investigated as a possible source of the required effluent. This facility of the Town of Colonie, New York serves approximately 80 families and utilizes extended aeration as the treatment method. The proximity of the site to the laboratory was a consideration in its favor, but the organic carbon concentration in the effluent and a critical appraisal of the operation of the plant and the form of treatment employed raised doubts as to its suitability for use in the study.

The total organic carbon concentration of the sample collected from this facility is noted in Table 3. This high concentration, 21 mg/l, was not in keeping with the requirements imposed by the first criterion and also gave pause in that it was indicative of possible operational difficulties. Although a preliminary chromatographic analysis was performed, Figure 3, the facility, due to its noncompliance with either of the criteria set forth above, was deleted from the list of possible sources of effluent.

Coxsackie Reformatory wastewater treatment facility. The second effluent investigated for possible use in the study was that produced by the trickling filter plant located at the Coxsackie Correctional Facility, Coxsackie, New York. The carbon concentration of the original sample collected, 11 mg/l, gave hope that the facility would produce the type of effluent required. Critical examination, however, proved otherwise. Subsequent samples proved to be high in organic carbon concentration and heavily loaded with particulates, which led to much difficulty in filtration.

However, the above was not the sole reason for discontinuance of the use of this effluent. It was felt also that its lack of accord with the second criterion regarding applicability for large scale systems should militate against its use in the study. Thus, as with the extended aeration facility, the Coxsackie installation was removed from contention. As noted previously for the extended aeration facility, preliminary chromatographic work was performed on the effluent and such chromatographic data are presented in Figure 4.

Batavia water pollution control plant. The final plant investigated as a possible source for the effluent for the study was the 2.5 mgd conventional activated sludge facility located at Batavia, New York. Figure 5 represents a schematic diagram of the treatment facility.

Table 3. Total Organic Carbon Cencentration of Membrane Filtered Wastewaters

Facility	Date Sampled	Total Organic Carbon mg/1
Eastern-Western	9-30-68	21
Coxsackie	10-18-68	11
Batavia	12-14-68	8
	1-22-69	7
	3-17-69	9

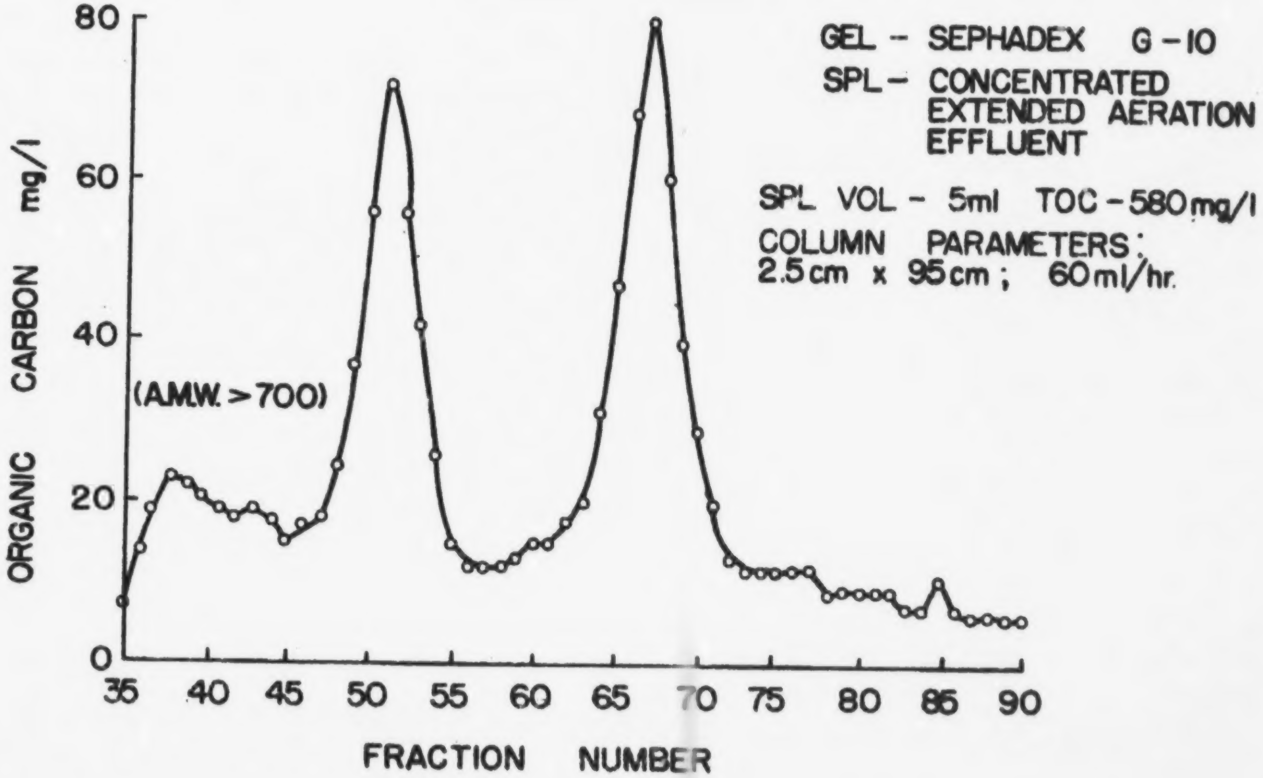


Fig. 3 ELUTION DIAGRAM RESULTING FROM THE FRACTIONATION OF CONCENTRATED EASTERN-WESTERN TREATMENT PLANT EFFLUENT ON SEPHADEX G-10.

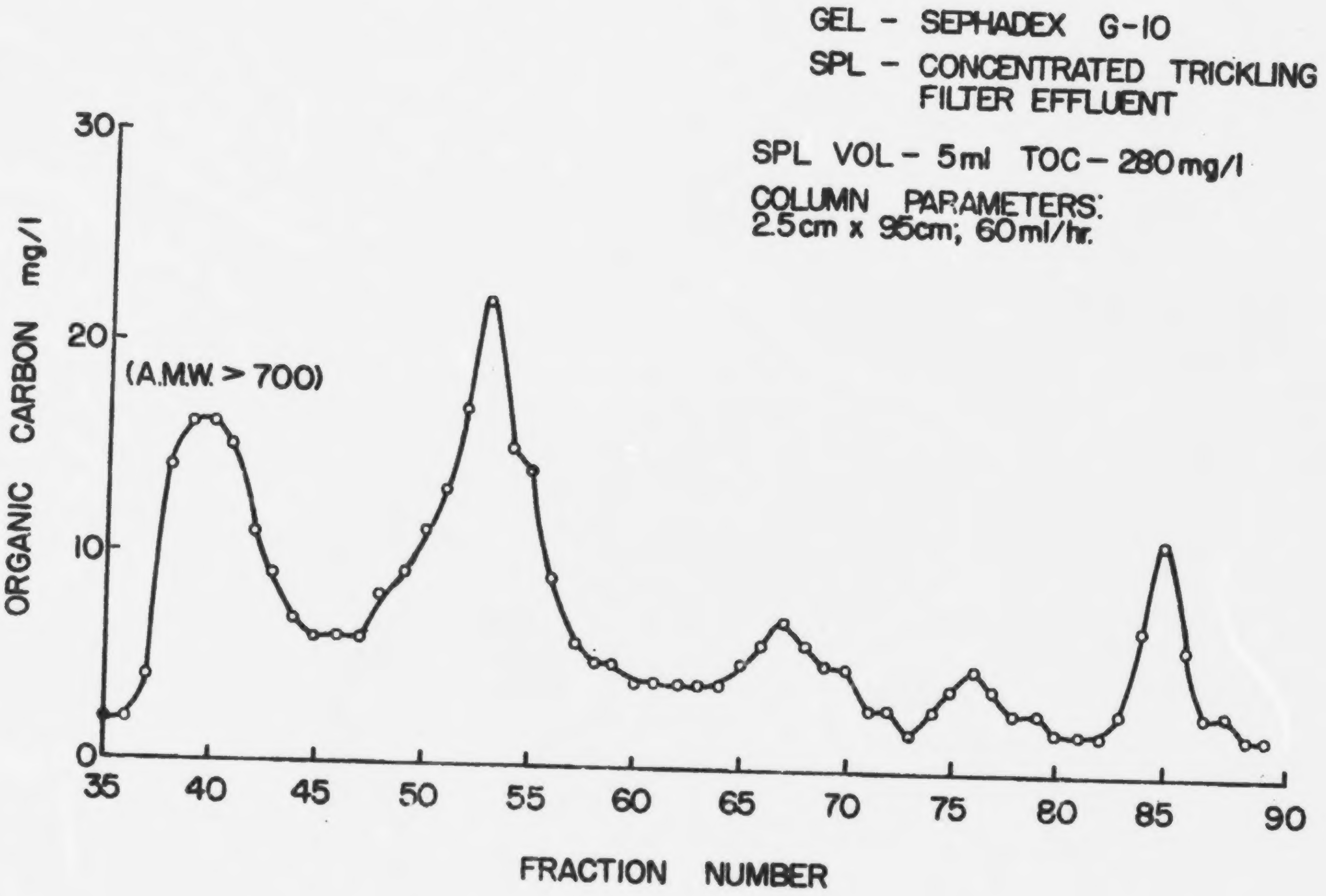


Fig. 4 Elution diagram resulting from the fractionation of concentrated Coxsackie treatment plant effluent on Sephadex G-10.

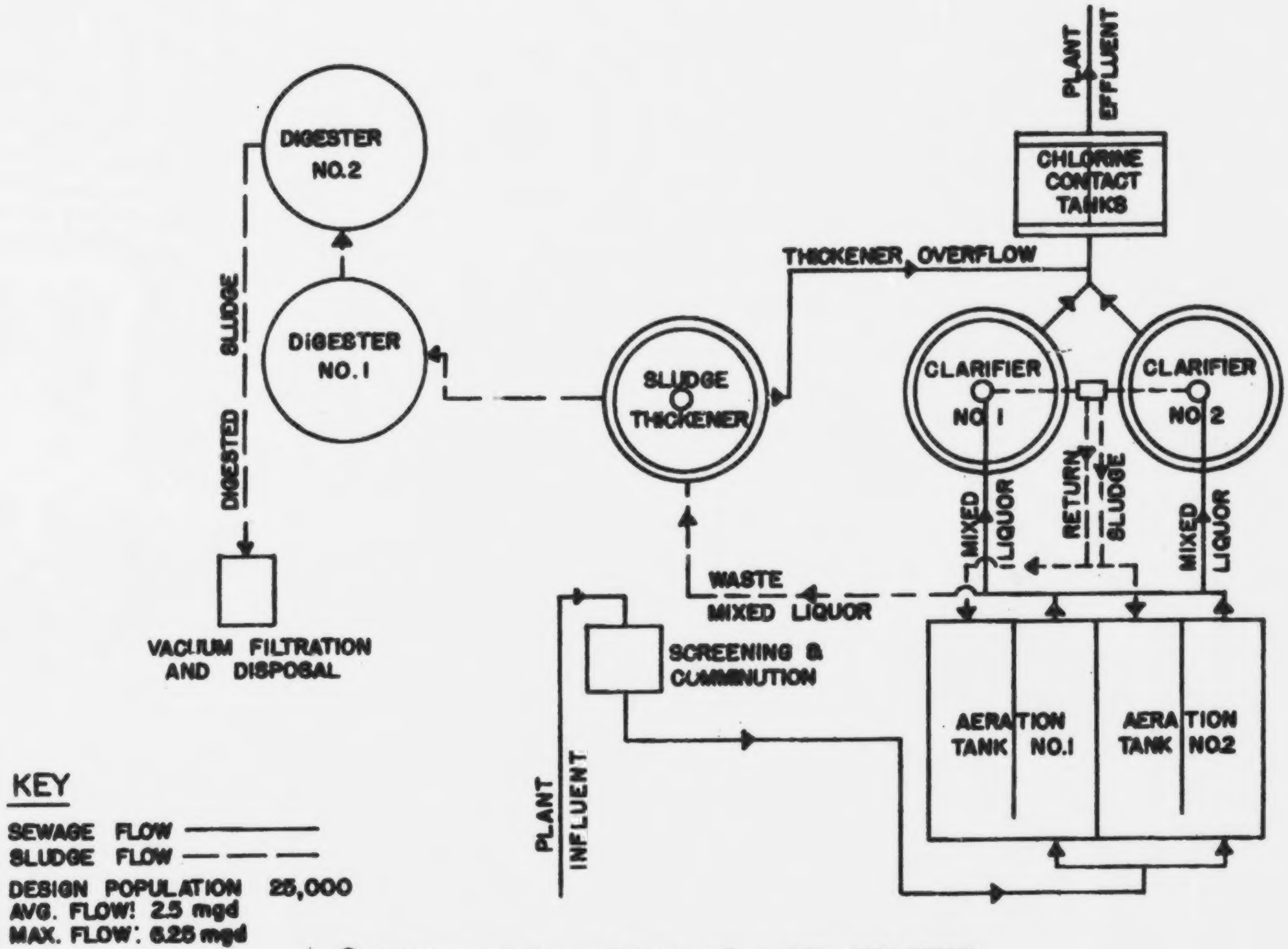

PLANT EFFLUENT
CHLORINE CONTACT TANKS
THICKENER OVERFLOW
DIGESTER NO.2
DIGESTER NO.1
DIGESTED SLUDGE
SLUDGE THICKENER
CLARIFIER NO. 1
CLARIFIER NO. 2
MIXED LIQUOR
RETURN SLUDGE
MIXED LIQUOR
WASTE MIXED LIQUOR
VACUUM FILTRATION AND DISPOSAL
SCREENING & COMMINUTION
AERATION TANK NO.1
AERATION TANK NO.2
PLANT INFLUENT
KEY
SEWAGE FLOW
SLUDGE FLOW
DESIGN POPULATION 25,000
AVG. FLOW: 2.5 mgd
MAX. FLOW: 6.25 mgd

Fig. 5 Schematic flow diagram of water pollution control facility, Batavia, New York.

Noted in Table 3 are carbon concentrations of three representative samples collected from this facility. As can be seen from the data presented therein, the total organic carbon concentrations of the effluents fully satisfied the requirements of criterion a. In addition, as was not the case with Eastern-Western or the Coxsackie Reformatory facility, operational data were available for this facility. Table 4 represents monthly averages of the BOD and suspended solids loadings and removals effected by the plant during the year 1968. From a review of these data, it is quite readily descernible that the facility was well operated and did produce an acceptable effluent.

This information, coupled with the fact that conventional activated slude treatment is well suited to wide-scale usage on a regional basis as required by the second criterion, prompted the selection of this facility as the sampling source. The chemical characteristics of the 1-22-69 effluent used in the study are shown in Table 5.

Freeze-drying. Depicted in Table 6 are data regarding recovery of total organic carbon resulting from both thin film rotary evaporation methods and freeze-drying concentration. The data contained therein clearly indicate that the freeze-drying method produced a higher recovery of organic carbon in the soluble portion. No data were garnered as to the carbon content of the acid wash for the thin film system so that a comparison on an overall recovery basis is not possible; but based upon the data relative to the recovery of the soluble organic component, however, it is obvious that freeze-drying was the more efficacious of the two methods in this particular instance.

No question has ever been raised as to the efficacy of freeze-drying in regard to retaining the basic character of a sample concentrated by this method. However, to test the validity of this basic assumption with particular reference to wastewater, near the conclusion of the study an untreated sample of wastewater from Clifton Knolls, New York was concentrated by the freeze-drying method and both the unconcentrated and concentrated portions subjected to fractionation on Sephadex G-15. The total organic carbon concentrations of the fractions resulting from the concentrated sample were measured with the Beckman Model 315 carbonaceous analyzer and those produced by the unconcentrated sample were measured by the

Table 4. Selected Operational Data for Batavia Wastewater Treatment Plant, 1968

Month	Average Flow mgd	Average Influent Suspended Solids lbs/day	Average Influent BOD lbs/day	Percent Suspended Solids Removed	Percent BOD Removed
January	2.4	4760	5627	90	88
February	2.5	3670	3936	89	89
March	3.3	5182	5447	88	88
April	2.3	3670	4899	84	86
May	3.3	6262	7370	83	84
June	2.7	4644	7383	87	85
July	2.3	3064	6777	91	89
August	1.9	3418	4900	88	85
September	1.5	3315	3293	88	89
October	1.3	3007	2566	90	89
November	2.6	4530	5240	87	88
December	2.6	4321	4313	87	84
Year Average				88	87

Table 5. Chemical Analysis of Membrane Filtered Batavia Effluent, Collected 1-22-69

Constituent	Concentration*
Cl	200.00
Total Alkalinity as $CaCO_3$	327.00
NH_4 as N	8.00
Organic N as N	12.00
NO_2 as N	0.20
NO_3 as N	1.20
Fe (total)	0.41
Total PO_4 as PO_4	7.06
Orthophosphate as PO_4	6.95
SO_4	116.00
Ca as $CaCO_3$	360.00
Mg as $CaCO_3$	40.00
Na	116.00
K	7.00
Organic Carbon	7.00
pH	7.90

* all expressed in mg/l except for pH

The above analyses with exception of Organic Carbon were performed by the Division of Laboratories and Research New York State Health Department.

Table 6. Comparison of Recovery of Organic Carbon, Thin Film Rotary Evaporation vs Freeze-drying

Effluent	Concentration Method	Total Organic Carbon In Original Sample (mg)	Total Organic Carbon in Soluble Organic Component (mg)	Percent Recovery Soluble Organic Component	Total Organic Carbon in Acid Soluble Component	Percent Recovery Total
Eastern-Western STP	Thin-Film Rotary Evaporation	84	57	68	----	----
Coxsackie Reformatory STP	Thin-Film Rotary Evaporation	33	20.1	61	----	----
Batavia STP 1-22-69	Freeze-drying	213.5	190	89.0	25	100
Batavia STP 3-17-69	Freeze-drying	360	284	79	14	83
Batavia STP 12-14-68	Freeze-drying	42	38	92	2.5	97

MSA LIRA Model 20 low level carbon analyzer. The resulting chromatograms are depicted in Figure 6. The data developed clearly indicate that the concentration of a wastewater sample by the freeze-drying method neither affects the basic character of the sample, nor leads to selective carbon losses in that the respective peaks are changed only in height, representative solely of a change in concentration.

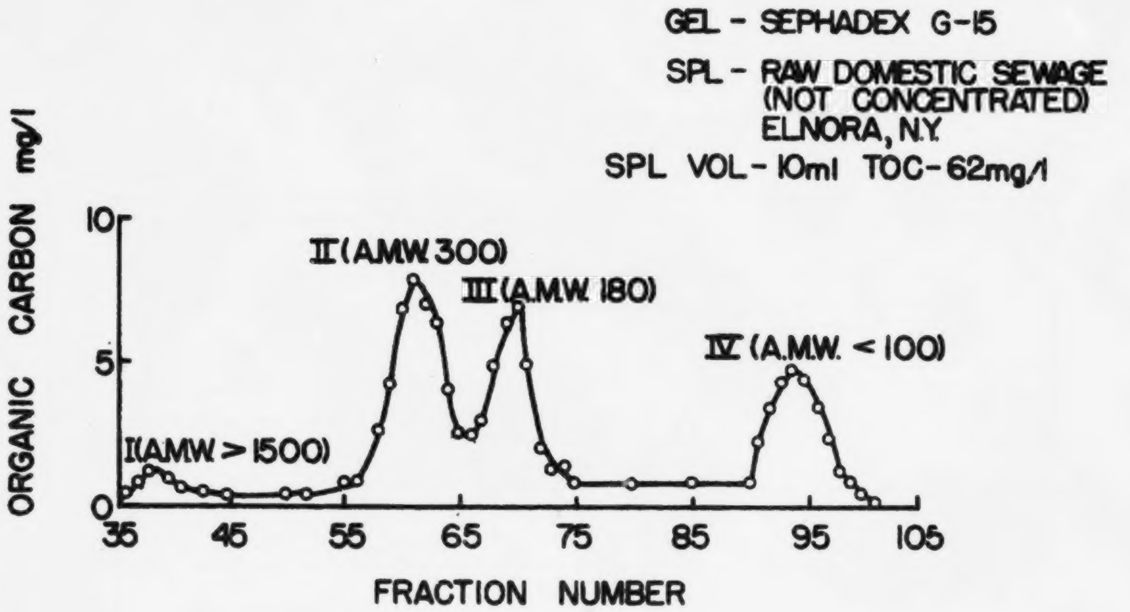
Based on the above information, it was concluded that the concentrated Batavia effluent and the resulting chromatographic fractions were in all aspects truly representative of the organic matter extant in the unconcentrated effluent. Additional data on this and other concentration procedures are found in Appendix 1.

2. Fractionation

The original method of choice for the fractionation of the organic matter in the concentrated wastewater was the separation of organic from inorganic components in the concentrate via ion exchange resins follows by fractionation of the organic components by gel permeation chromatography. Preliminary experimentation using the ion exchange procedure and an unconcentrated wastewater proved that the method would be totally unsatisfactory. Although the removal of the inorganic fraction was successful, a major portion, greater than 50%, of the effluent organics was lost due to adsorption onto the resin.

Therefore the ion exchange step originally posited was elimated and the fractionation procedure narrowed to gel permeation chromatography alone. The chromatography setup used is shown in Figure 7. Standard chromatographic columns 2.4 x 100 cm, fitted with upflow adaptors to optimize the resolution of the fractionation procedure were employed (Pharmacia Fine Chemicals). This apparatus was operated in the cold room.

At first it was felt that possibly Bio-Gel, a cross-linked polyacrylamide manufactured by Bio-Rad Corporation of Richmond, California, would be satisfactory for use as the gel in the procedure. However, investigation showed that Bio-Gel would be unsatisfactory. Attention was then focused on the Sephadex gels manufactured by Pharmacia Fine Chemicals. The Sephadex gels finally used in the investigation were G-10, G-25, and G-50. Data relative to these gels may be found in Table 7.



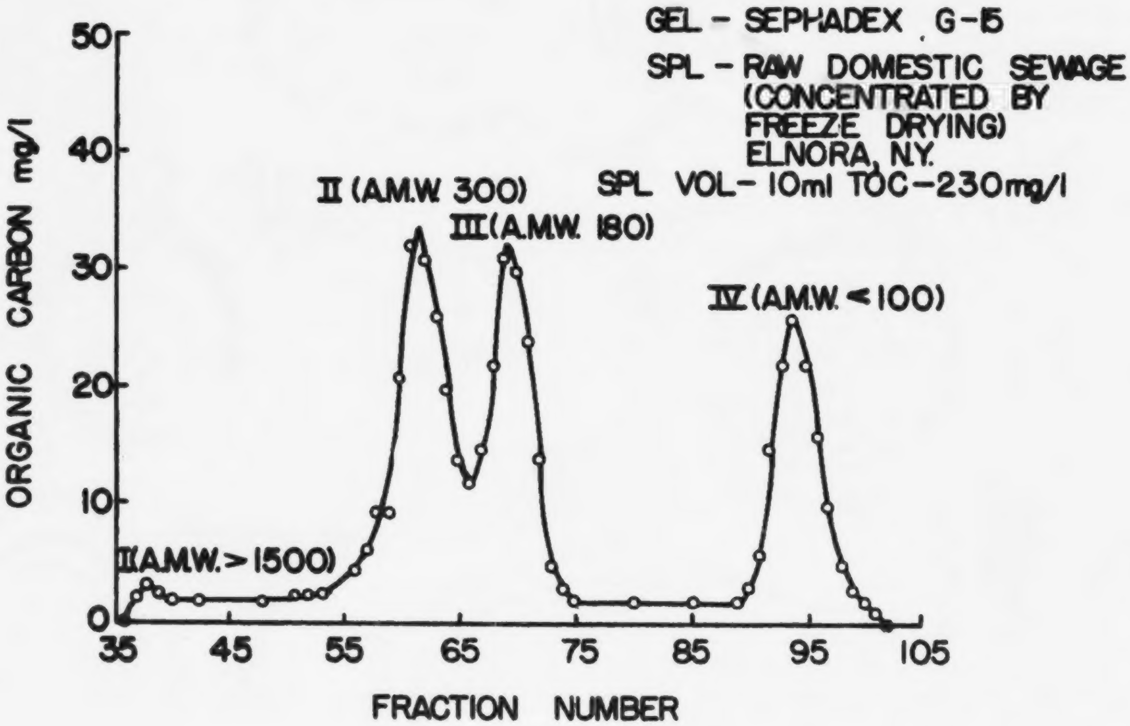


Fig. 6 ELUTION DIAGRAM OF THE FRACTIONATION OF UNCONCENTRATED AND CONCENTRATED SAMPLES OF RAW DOMESTIC SEWAGE FROM ELNORA, NEW YORK ON SEPHADEX G-15.

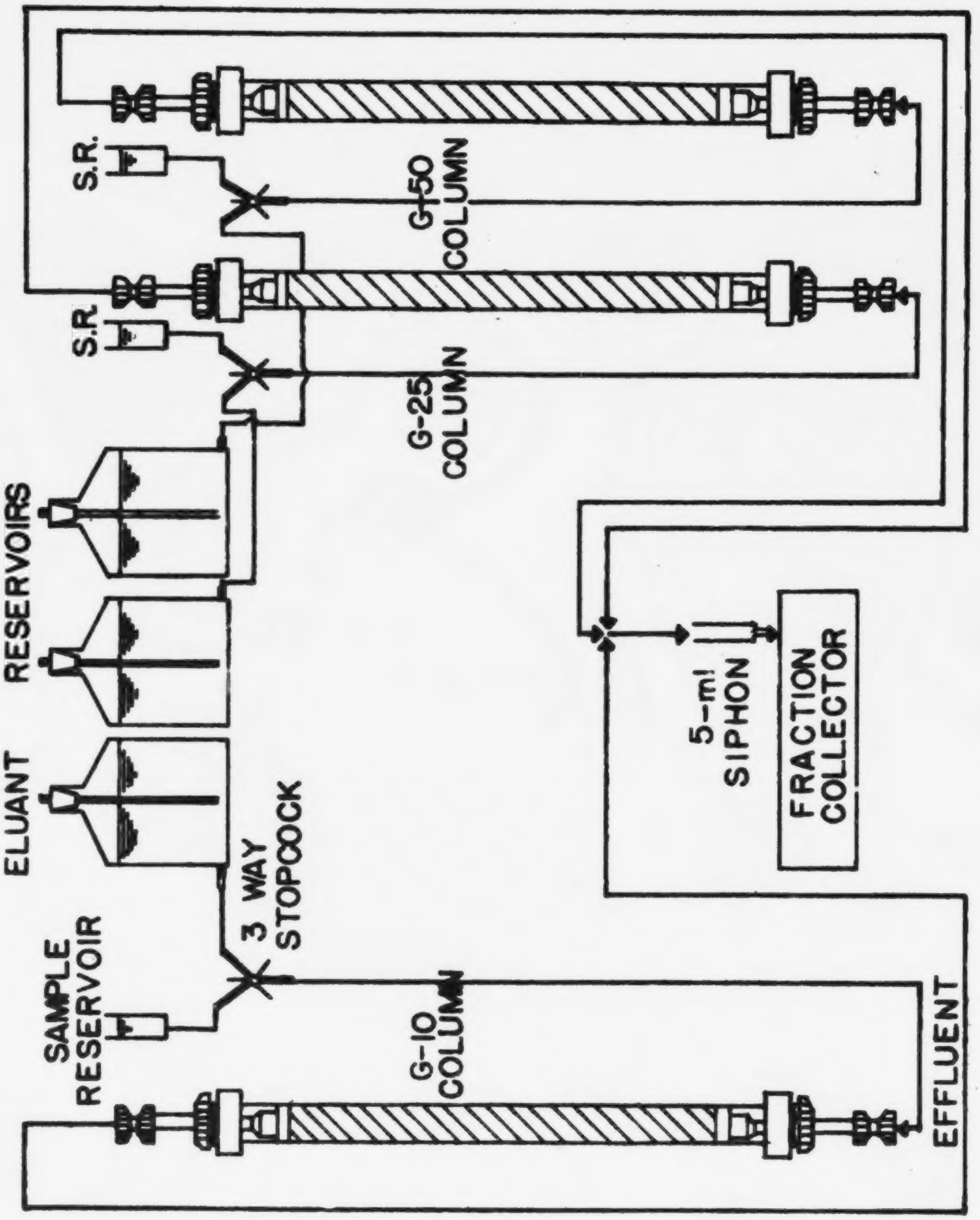

SAMPLE RESERVOIR
3 WAY STOPCOCK
ELUANT
RESERVOIRS
S.R.
S.R.
G-10 COLUMN
G-25 COLUMN
G-50 COLUMN
EFFLUENT
5-ml SIPHON
FRACTION COLLECTOR

Fig. 7 GEL CHROMATOGRAPHY APPARATUS.

Table 7. Properties of Dextran Gels Sephadex, After Determann (20)

				Approximate Separation Range			
Type	Particle Size (dry; in u)	Water Regain (ml/g)	Gel Bed (ml/g)	Peptides and Globular Proteins		Dextran Fractions	
G-10	40-120	1.0 ± 0.1	2-3	up to	700	up to	700
G-15	40-120	1.5 ± 0.1	2.5-3.5	up to	1,500	up to	1,500
G-25, coarse	100-300	2.5 ± 0.2	4-6	1,000	5,000	100	5,000
G-25, medium	50-150	" "	"	"	"	"	"
G-25, fine	20-80	" "	"	"	"	"	"
G-50, coarse	100-300	5.0 ± 0.3	9-11	1,000	30,000	50	10,000
G-50, medium	50-150	" "	"	"	"	"	"
G-50, fine	20-80	" "	"	"	"	"	"
G-75	40-120	7.5 ± 0.5	12-15	3,000	70,000	1,000	50,000
G-100	40-120	10.0 ± 1.0	15-20	4,000	150,000	1,000	100,000
G-150	40-120	15.0 ± 1.5	20-30	5,000	400,000	1,000	150,000
G-200	40-120	20.0 ± 2.0	30-40	5,000	800,000	1,000	200,000

Column packing. To assure that the chromatographic analyses would be successful, the columns were packed with the utmost care. The gel was swelled for a minimum of 24 hours in the cold room and the column filled, prior to packing, with glass distilled water. This water had been stored in the cold room for the same length of time as the swelling gel. The outlet of the column was shut off and a funnel fitted with a rubber stopper was placed into the top of the column and this filled partly with the glass distilled water.

In one pass sufficient gel to fill the column was introduced into the funnel and continuously stirred while the gel settled into the column. After a bed height of approximately 5 cm had been established, the outlet of the column was opened slightly to enable passage of water out of the column and thus increase the rate of sedimentation of the gel into the column. After the final bed height had been established, the outlet was shut off and the funnel removed. The column above the bed was allowed to remain filled with water to assure that the top of the bed did not go dry.

The upflow adaptor to be inserted in the top of the column was connected to the eluent reservoir bottle and the reservoir outlet opened. In this way water was continuously allowed to flow through the adaptor. The adaptor was then gently introduced into the top of the column through the head of water above the column bed with care, so as to assure that no air bubbles were trapped beneath or on the side of the adaptor gasket. When the adaptor reached the top of the bed the gasket control was tightened securely and the column top closed.

The column was then inverted. This inversion step was devised because previous experience in packing columns had shown that if the eluent reservoir connection was made to the bottom adaptor, the gel bed would rise off the column base. By inverting the column, this difficulty was avoided. After the connections had been secured the eluent was allowed to run through the column for 48 hours prior to any sample application. The eluent used throughout the study was glass distilled water.

Sample application. As shown in Figure 7 the eluent and sample reservoirs were connected to a three-way stopcock, which connection allowed the addition of either sample, up to 10 ml, or eluent. Prior to the actual fractionation of a sample, approximately 2 to 3 ml of the sample was run into the column, followed with a bed volume of eluent, which was run to waste. This initial step was taken to assure (1) that mixing of dissimilar samples did not occur in the sample feed line leading to the three-way stopcock, and (2) that the sample volume registered by the pipette used as the sample reservoir truly represented the volume applied to the column. The sample to be fractionated was drawn up into a 10 ml syringe, and then discharged through a Swinnex membrane filter (0.45 u) into the 10 ml pipette which served as the sample reservoir. The stopcock was then adjusted so that the sample could flow on to the column bed. After sample introduction had been completed, the stopcock was switched to the eluent position. Careful checks were made to insure that no air bubbles were present in the feed line leading to the column.

The column effluent was collected in 5 ml portions, using a 5 ml siphon and an LKB Radirac fraction collector. Several positions were available for the placement of the eluent reservoir bottle so that varying flow rates could be obtained. This eluent reservoir placement was especially critical for the more loosely cross linked gels, G-25 and G-50.

Column standardization. It has been demonstrated that over a wide range of elution volume of an organic compound, that volume in which the maximum concentration of the compound is obtained, is directly proportional to the log of the molecular weight of the compound (3, 4, 20, 31, 71).

To determine the apparent molecular weight of the fractions produced by the application of an unknown sample onto a Sephadex column, the column must first be standardized. The standards used for G-10 with an upper exclusion limit of molecular weight 700 were solutions containing glucose (molecular weight 180), sucrose (molecular weight 342), raffinose (molecular weight 594.5) and egg albumin (molecular weight 45,000).

Blue dextran, consisting of a dyed dextran with a molecular weight of approximately 2 million was also used to standardize the G-10. This dextran was used specifically to determine the degree of band broadening experienced during the passage of a sample through the columns.

The standard solutions were applied to the G-10 column and the organic carbon concentrations of the 5 ml fractions analyzed with the Beckman carbonaceous analyzer. A plot was then developed of milligrams per liter carbon versus fraction number, with each fraction number of maximum concentration of each of the organic compounds in the standard solution taken as the elution volume of that organic compound. Based on these data a plot of fraction number versus log molecular weight was subsequently produced, thus providing a standard curve for the G-10 column. Using the elution volumes of the fractions of an unknown sample, one could then enter the curve and deduce an apparent molecular weight.

For the G-25 and G-50 standardizations, the standard solution contained egg albumin (molecular weight 45,000). The points chosen to develop the standard curves for the G-25 and G-50 columns were the theoretical end of the bed calculated from data provided by the Sephadex manufacturers and the elution volume of the egg albumin, which denoted the void volume of the column.

Carbon analysis. The organic carbon in each of the 5 ml fractions was determined using the Beckman carbonaceous analyzer. A 20 microliter sample of the fraction was removed with a syringe and discharged into the effluent port of the analyzer. The sample then passed into a catalytic combustion chamber maintained at a temperature of 950°C. In this catalytic combustion chamber the carbonaceous portion of the sample is oxidized in the presence of a cobalt catalyst and pure oxygen carrier gas. This gas then carries the carbon dioxide produced from the combustion of the carbonaceous material and the water vapor out of the furnace. The water vapor is condensed in a trap and the carbon dioxide oxygen mixture remaining is then swept into the infrared analyzer.

The range of the instrument was calibrated with oxalic acid ($H_2C_2O_4 \cdot 2H_2O$). For a range between 0 mg/l and 100 mg/l C, the oxalic acid solution was made up initially in a concentration of 96 mg/l C, by dissolving 500 mg of the compound in distilled water and making the solution up to 1 liter volume. Portions of this solution were then diluted to produce standards containing 48 mg/l C and 24 mg/l C. At the commencement of each set of analyses using the carbon analyzer, the standards were injected and the results noted.

By judicious adjustment of the gain, the instrument can be made to read out the concentrations of carbon existing in the respective standards, and respond linearly in the range 0-100 mg/l.

Fraction compositing. To secure a sufficient volume of fractions for the subsequent biological analyses, the wastewater sample concentrate under investigation was fractionated several times on Sephadex G-10 and the similar resulting fractions composited. The fractions comprising the front from G-10 (molecular weight greater than 700), after the compositing step, were reconcentrated by freeze-drying and this concentration introduced onto the G-25 column.

The same procedure as followed with the G-10 column was also employed in compositing the similar fractions developed during the G-25 chromatographic analysis. The front from the G-25 column (molecular weight greater than 5,000) was reconcentrated and introduced onto the G-50 column, and the fraction appearing in the G-50 effluent composited.

The composite fractions were stored in the cold room in acid-washed serum bottles provided with syringe caps. Prior to storage, the fractions were analyzed for pH, conductivity and organic carbon concentration.

3. Chromatographic Data

Results with Bio-Gel P-2. The first attempts at fractionation were made with Bio-Gel P-2. Several difficulties, however, were encountered with the gel leading to its disuse after a short experimental procedure. It was first found that when blue dextran was applied to the gel, the color group of the dextran was adsorbed onto the gel material and no amount of eluent passed through the column would remove it from the gel bed. Subsequently a sample of concentrated effluent from the Eastern-Western facility was applied to the column. The resulting chromatographic data shown in Figure 8 showed only one peak approximately at the middle of the bed volume. This can be compared with the Sephadex G-10 chromatogram of the same effluent shown in Figure 3. No attempt was made to investigate the reasons for the failure of Bio-Gel P-2 to fractionate the wastewater organic components or to allow blue dextran to pass through without adsorption of the dextran color group. These occurrences provided the impetus

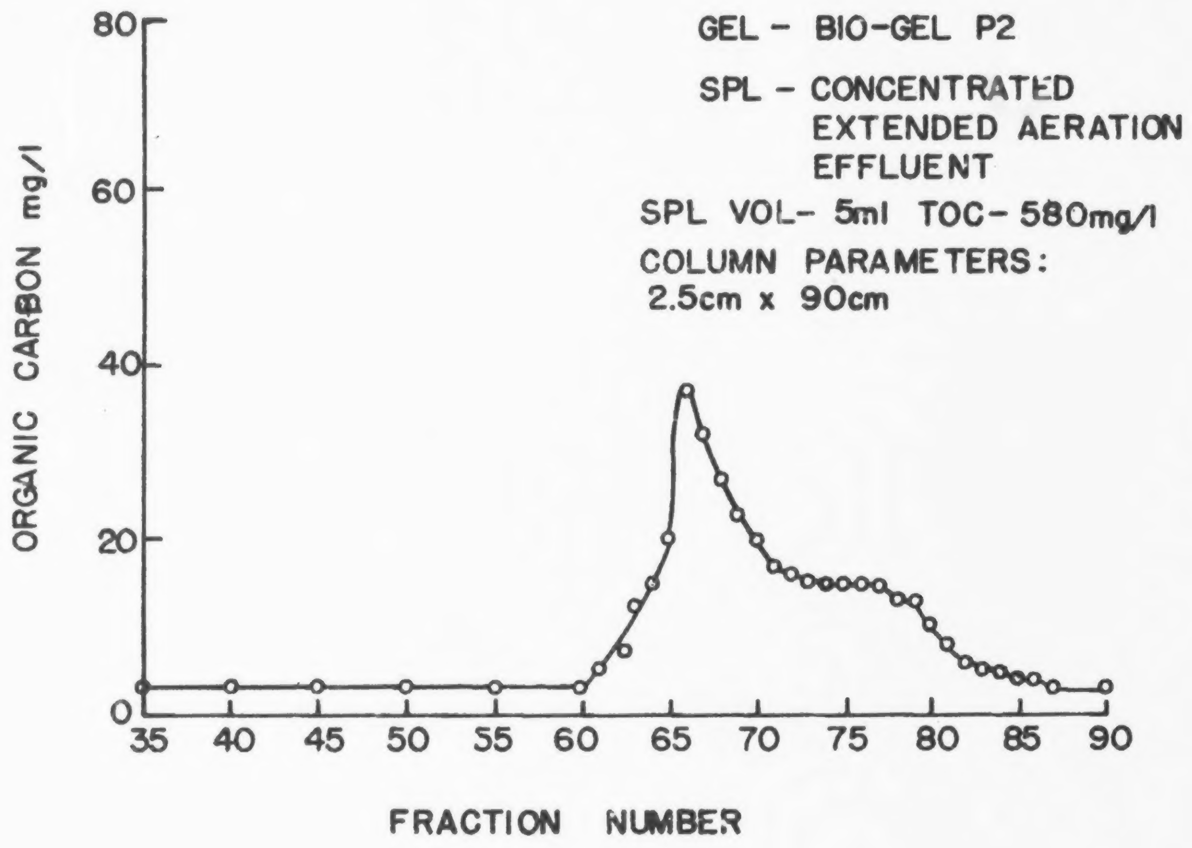


Fig. 8 Elution diagram of the fractionation of concentrated Eastern--Western treatment plant effluent on Bio-Gel P2.

for a switch to the Sephadex gels.

Standardization of Sephadex gels.

Sephadex G-10. The standard solution used with Sephadex G-10 was split into two portions...one containing egg albumin, raffinose and glucose and the other sucrose. The reason for this split was that originally a standard solution had been made up with all four compounds and the closeness of raffinose, glucose and sucrose in molecular weight did not allow for the resolution of these three compounds into separate and distinct peaks. The concentrations of these components in the standards used for the Sephadex G-10 gel column are given in Table 8. The chromatograms resulting from the application of standards 1 and 2 to the G-10 column are shown in Figure 9.

Based on the data presented therein, the elution volumes of the four standards and their respective molecular weights were used to produce a plot of elution volume versus log molecular weight for this specific column. A regression analysis was employed to obtain a line of best fit for the data. Using this plot, shown in Figure 10, one would then be able to assign an apparent molecular weight to the organic fractions produced upon passage of an unknown sample through the G-10 gel.

Sephadex G-25 and G-50 standardizations. Both G-25, with an upper exclusion limit of 5,000, and G-50, with an upper exclusion limit of 30,000, were standardized with a solution of egg albumin in glass distilled water at a concentration of 250 mg/l. The chromatograms resulting from the application of this standard to the G-25 and G-50 columns are shown in Figure 11.

Using the elution volume of the egg albumin and the theoretical end of the bed calculated from data provided by the manufacturer, the graphical representation of elution volumne versus log molecular weight was developed for the G-25 column. This standard curve is shown in Figure 12.

G-10 Chromatographic data. The 1-22-69 Batavia concentrate containing 380 mg/l of total organic carbon was concentrated by the freeze-drying method to a total organic carbon concentration of 1500 mg/l, with a resulting percentage recovery of 97%.

Table 8. Concentrations of Organic Compounds in Sephadex G-10 Column Standards

Compound	Standard Number	Concentration mg/l	Molecular Weight
Egg Albumin	1	250	45,000
Raffinose	1	687	594.5
Glucose	1	623	180
Sucrose	2	625	342

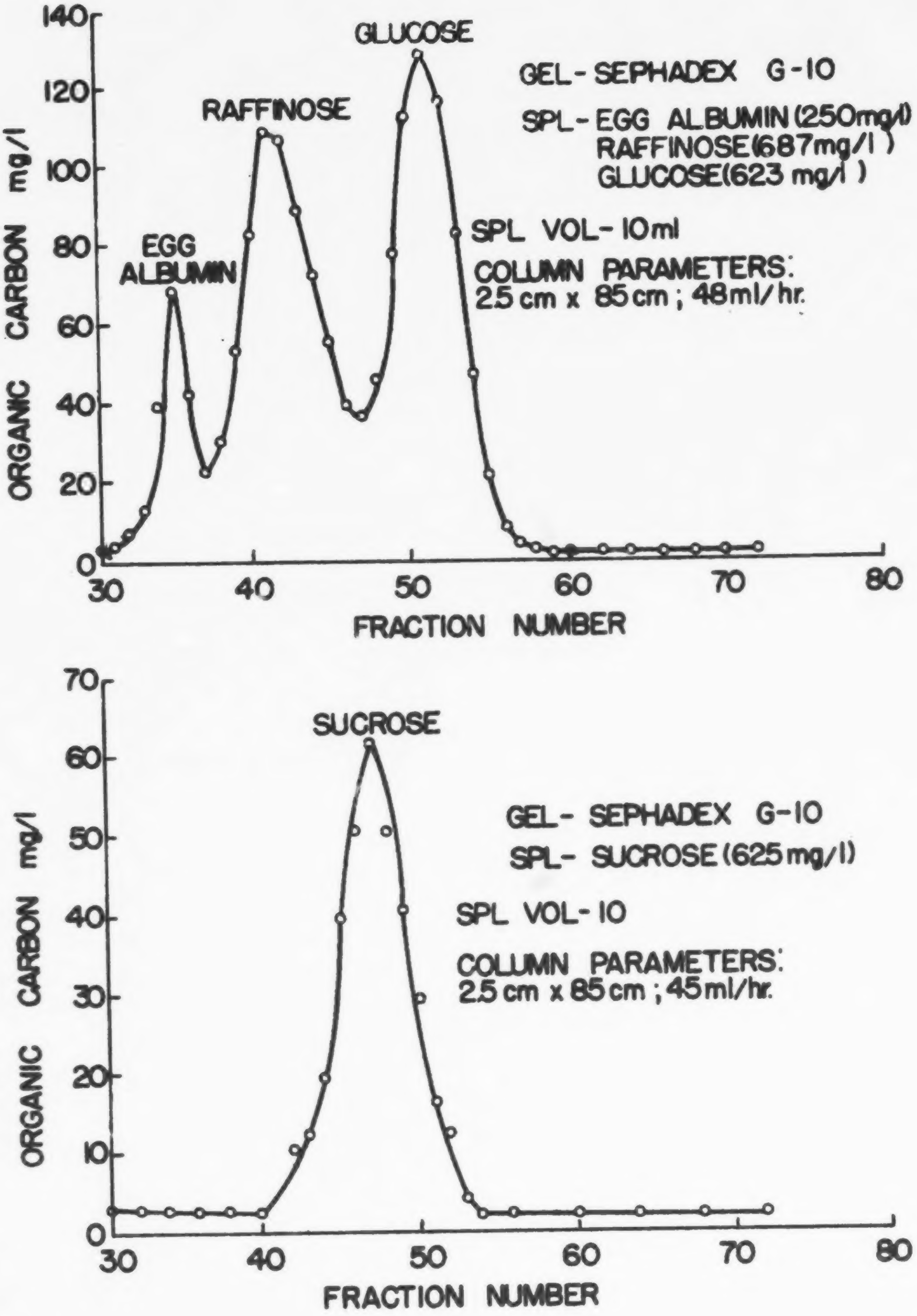


Fig. 9 GEL CHROMATOGRAPHY OF STANDARD SOLUTIONS 1 AND 2 ON SEPHADEX G-10.

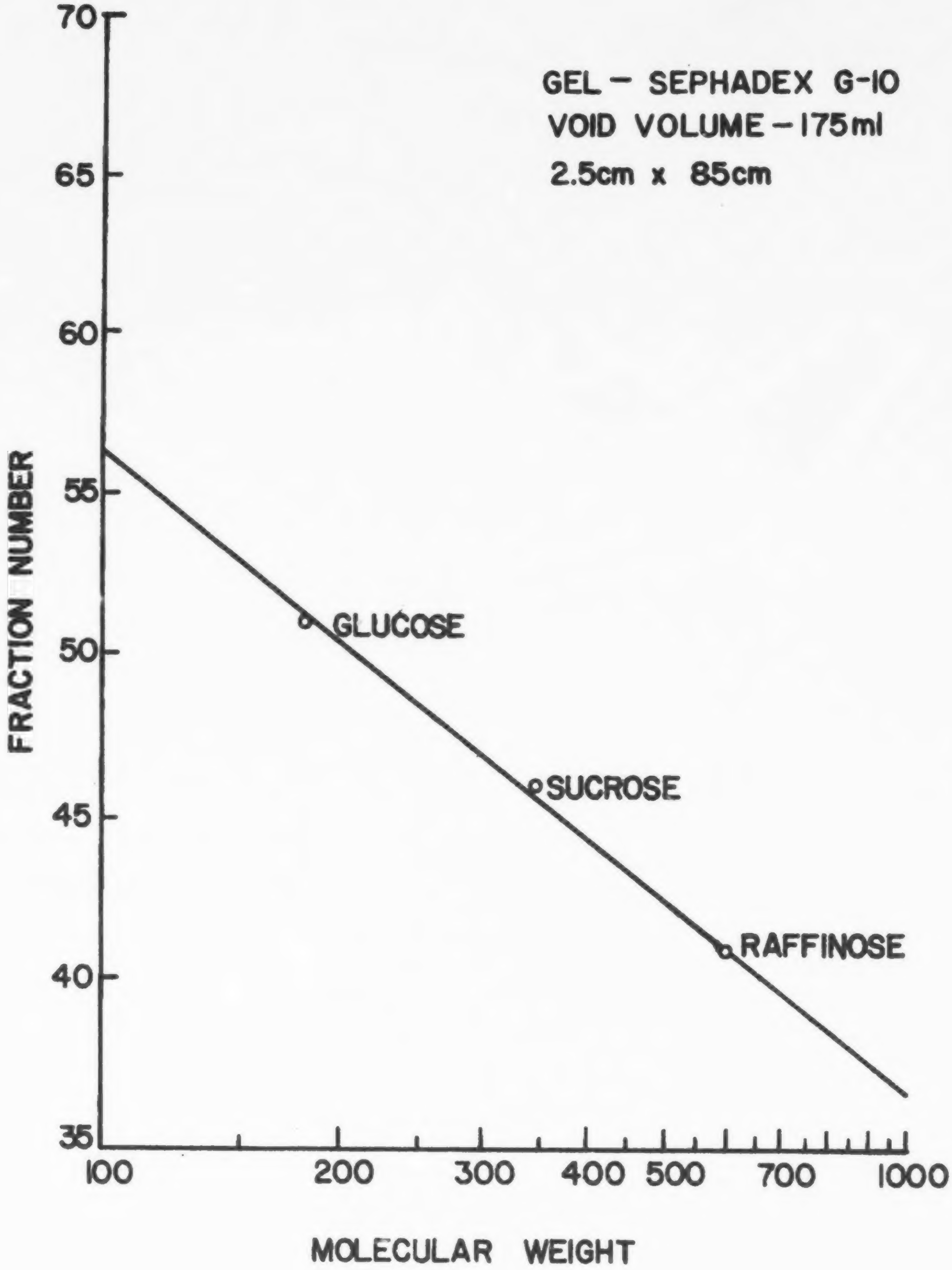


Fig. 10 GRAPHICAL REPRESENTATION OF THE RELATIONSHIP BETWEEN MOLECULAR WEIGHT AND ELUTION VOLUME FOR SEPHADEX G-10.

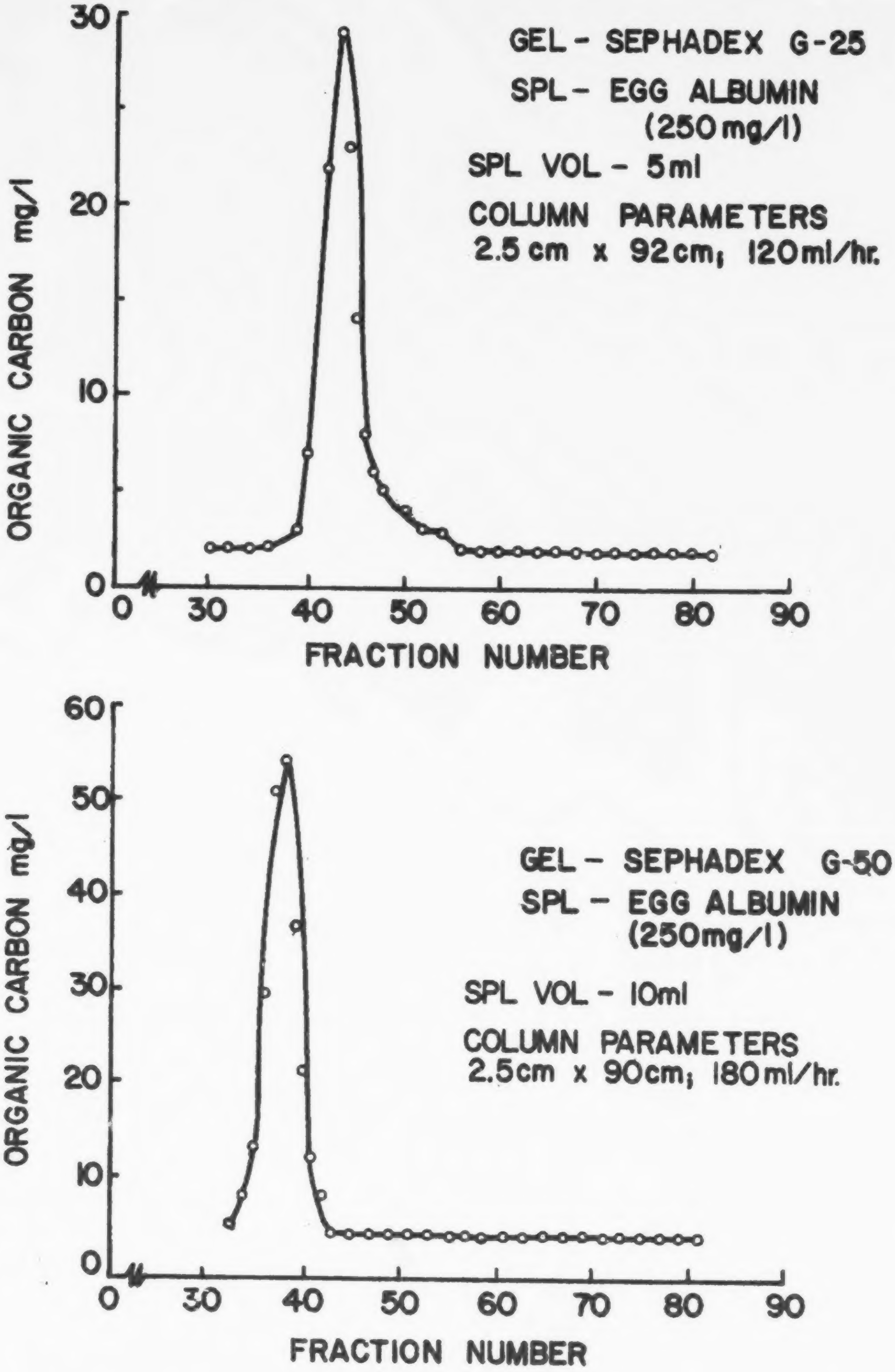


Fig. 11 GEL CHROMATOGRAPHY OF STANDARD EGG ALBUMIN SOLUTION ON SEPHADEX G-25 AND SEPHADEC G-50.

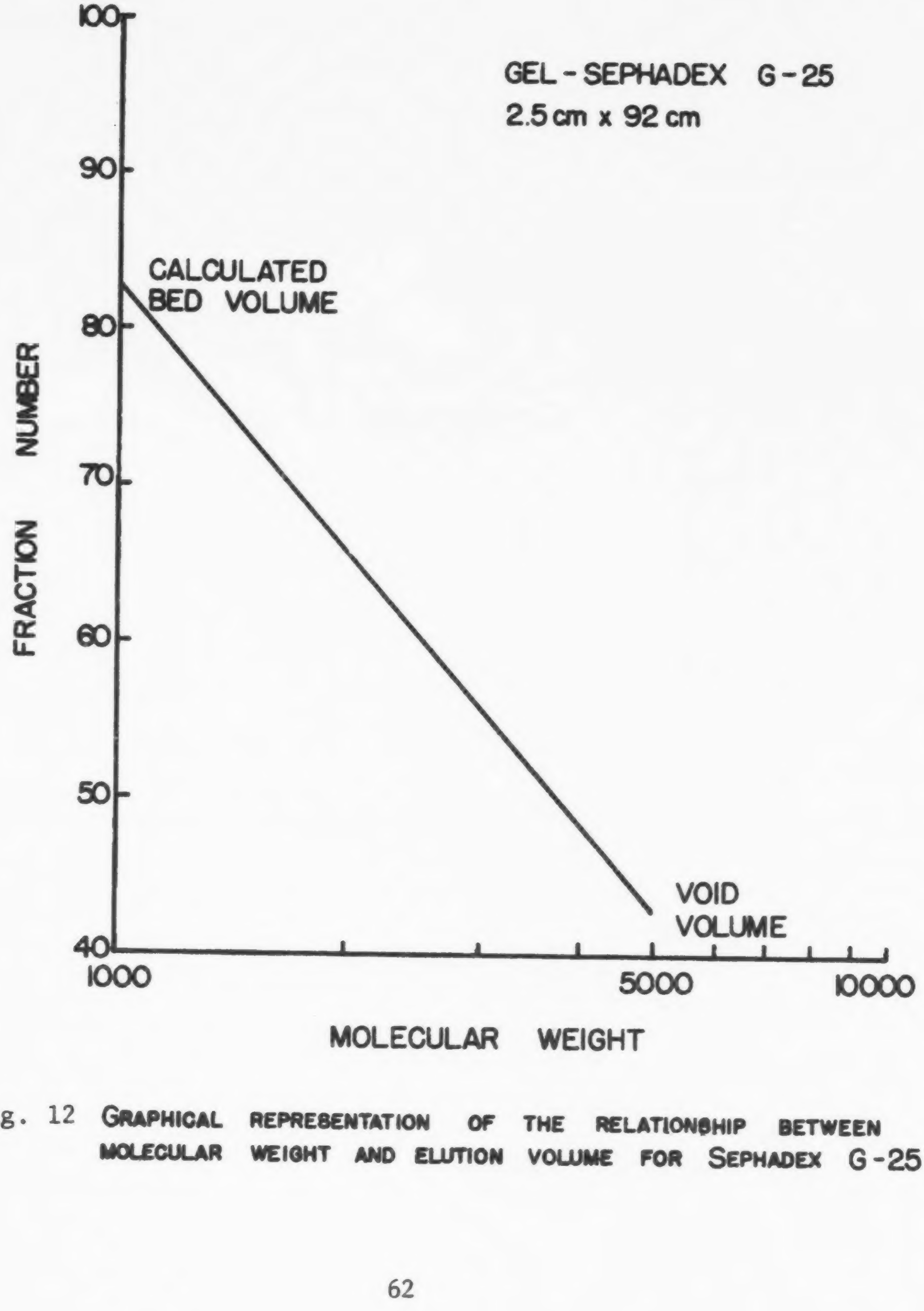


Fig. 12 GRAPHICAL REPRESENTATION OF THE RELATIONSHIP BETWEEN MOLECULAR WEIGHT AND ELUTION VOLUME FOR SEPHADEX G-25

The resulting concentrate was applied to the G-10 column in 10 ml passes. The graphs shown in Figure 13 represent the chromatograms produced as a result of the six runs on G-10 with the Batavia concentrate and are noted therein as Runs 1 through 6. The apparent molecular weights listed thereon are derived from the standard G-10 curve. Those organic fractions in the lower portions of the chromatogram have not been assigned a molecular weight as this area of a G-10 elution profile contain fractions with an indefinable molecular weight (20).

The data in Table 9 show the total organic carbon content in milligrams of each of the G-10 fractions for all six runs performed. The determination of the percentage of original effluent organic carbon that each fraction represented was based on these data. To ascertain the percentage of applied carbon represented by the various G-10 fractions, the values of carbon applied and total organic carbon content in each of the fractions are totaled and the percentages calculated from these totals. For example, the total organic carbon applied to the G-10 column in the six runs was 90 mg and of that amount 27.9 mg was found in the frontal peak, and thus the G-10 frontal peak, based on these data, was calculated to represent 31% of the organic carbon applied to the column.

Since no change had occurred in the character of the original sample through the concentration and chromatographic operations, this percentage should hold as a valid measure of the proportion of the G-10 frontal peak in the unconcentrated membrane filtered effluent. This fact has great significance, particularly with regard to the calculation of the percent of the effluent organic carbon content represented by the various fractions.

The fractions produced during the six runs on G-10 were composited in acid-washed serum bottles and stored in the cold room. The G-10 frontal peak was kept for further fractionation on Sephadex G-25 and the remainder of the peaks for biostimulation studies.

G-25 chromatographic data. The G-10 frontal peaks appearing in maximum concentration at fraction 35 noted as peak G-10-I were, subsequent to compositing, examined for total organic carbon concentration and reconcentrated by freeze-drying to

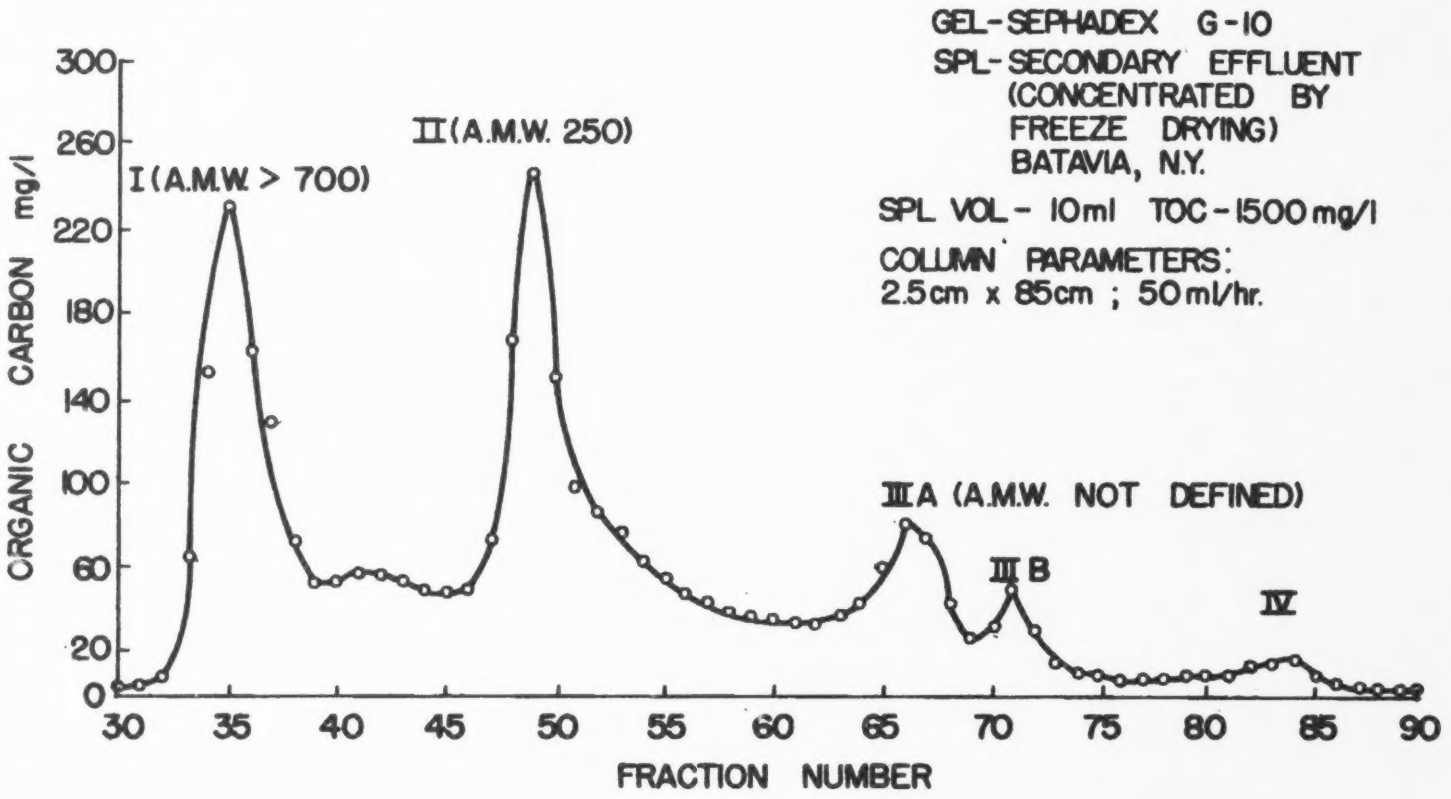


Fig. 13a ELUTION DIAGRAM OF THE FRACTIONATION OF CONCENTRATED BATAVIA EFFLUENT ON SEPHADEX G-10, RUN 1.

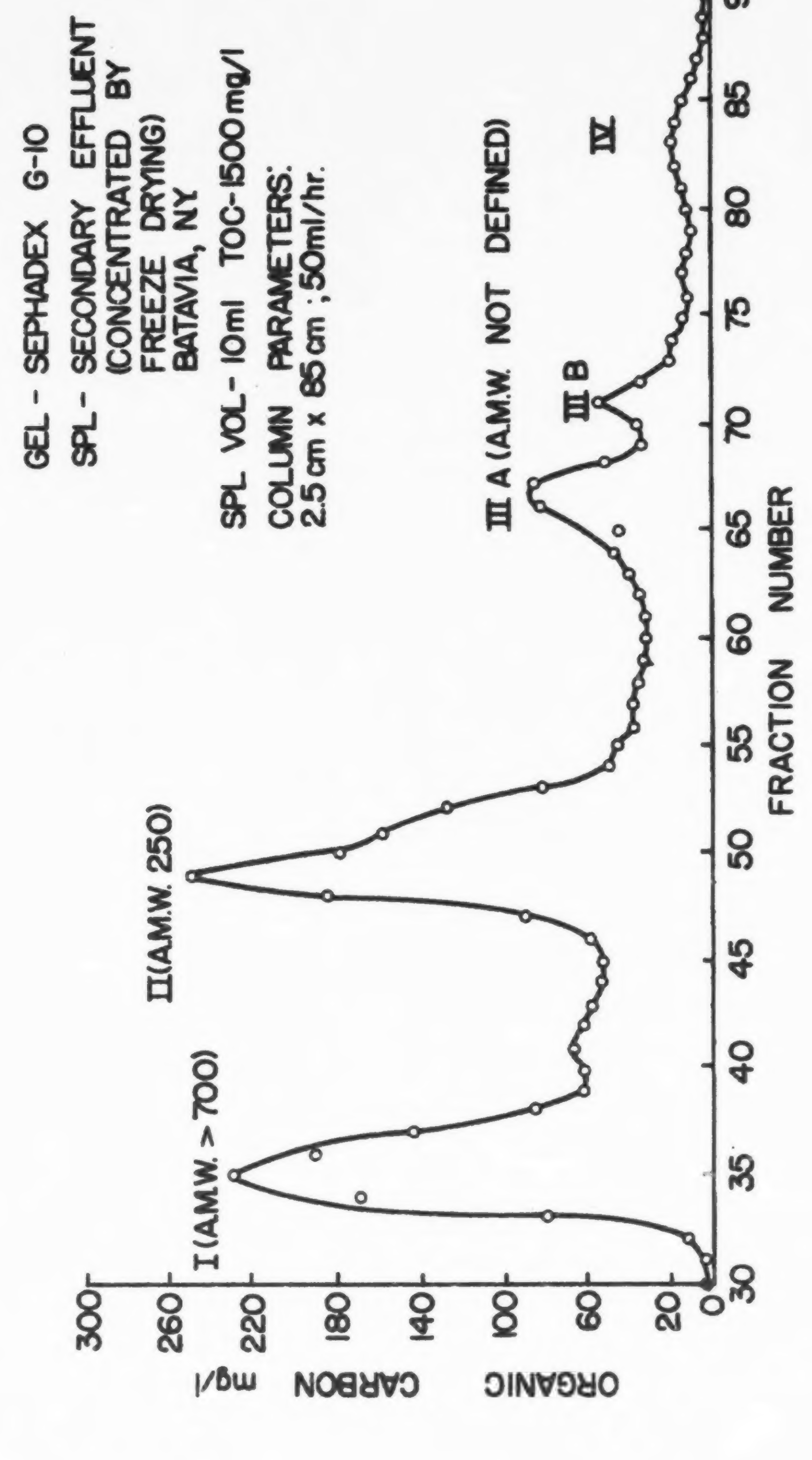


Fig. 13b Ellution diagram of the fractionation of concentrated Batavia effluent on Sephadex G-10, Run 2.

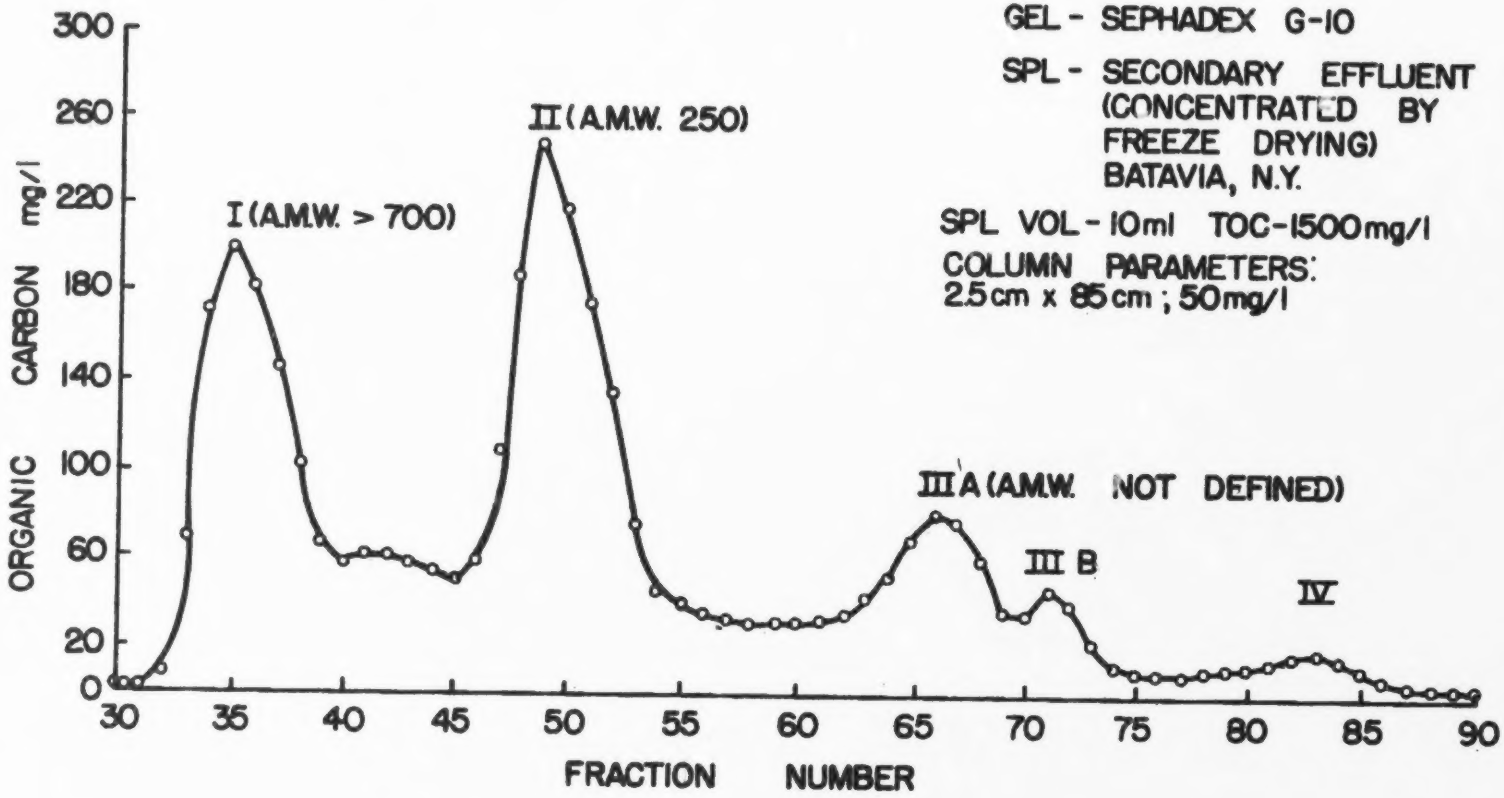


Fig. 13c Elution diagram of the fractionation of concentrated Batavia effluent on Sephadex G-10, Run 3.

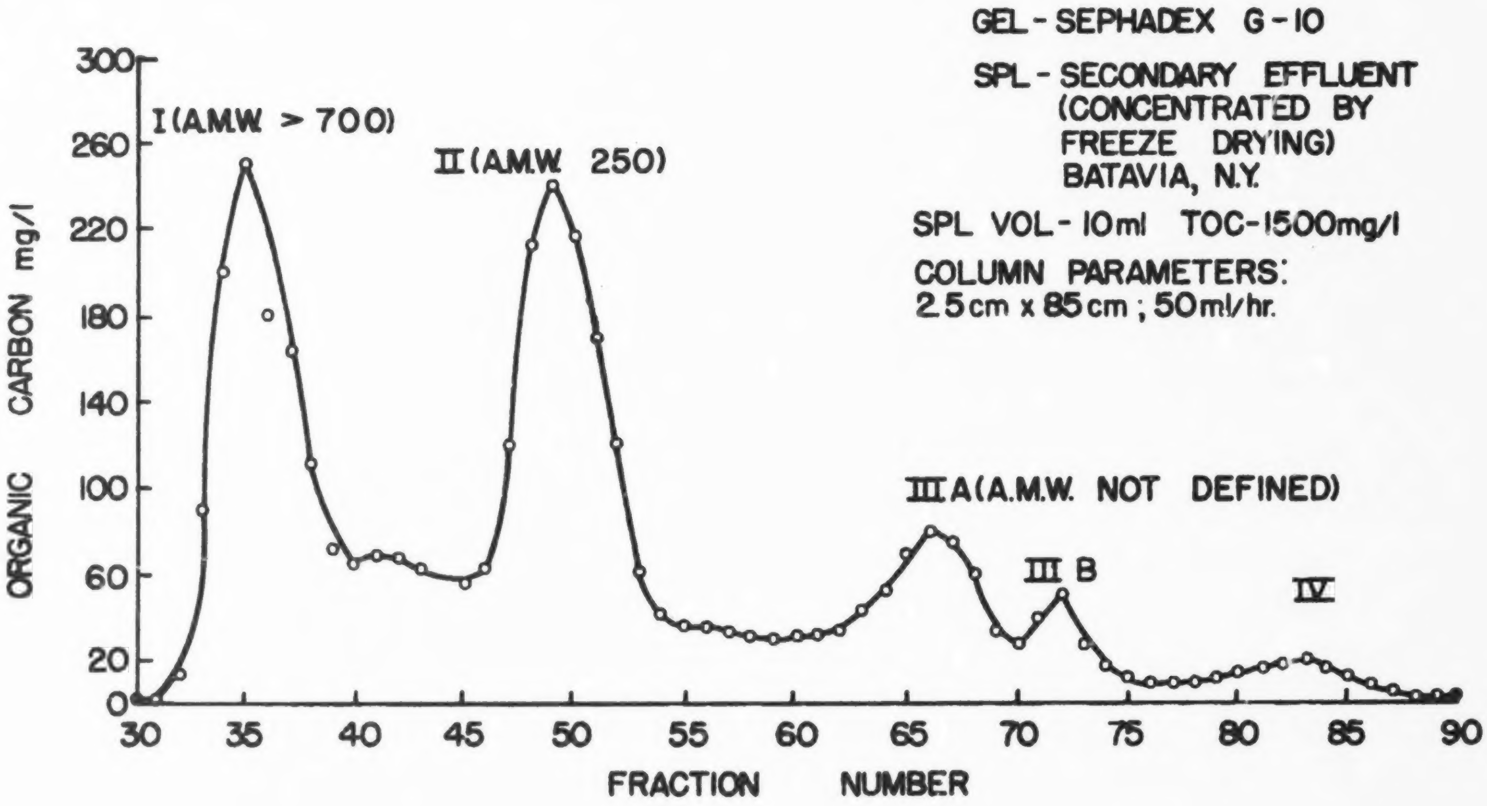


Fig. 13d Elution diagram of the fractionation of concentrated Batavia effluent on Sephadex G-10, Run 4.

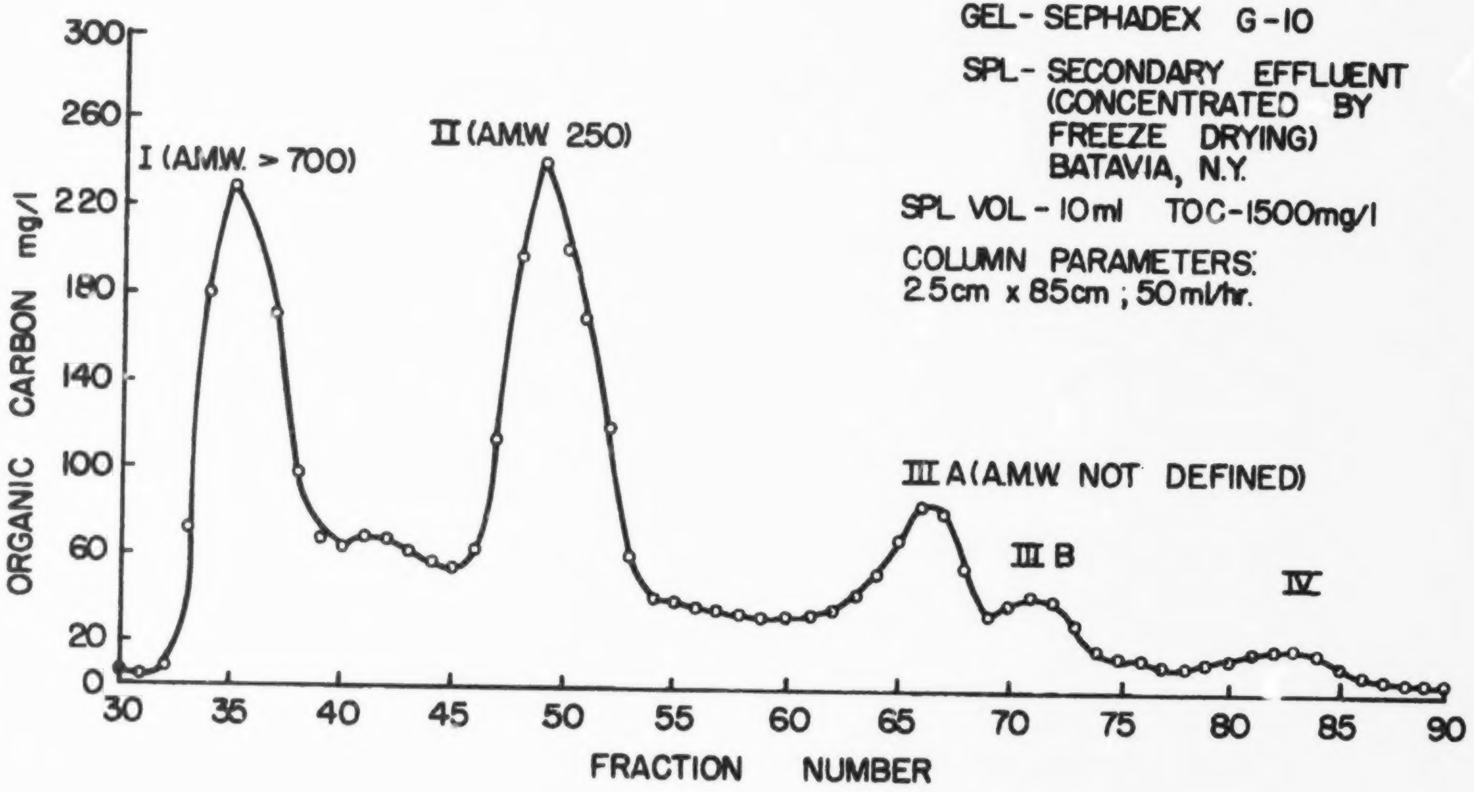


Fig. 13e ELUTION DIAGRAM OF THE FRACTIONATION OF CONCENTRATED BATAVIA EFFLUENT ON SEPHADEX G-10, RUN 5.

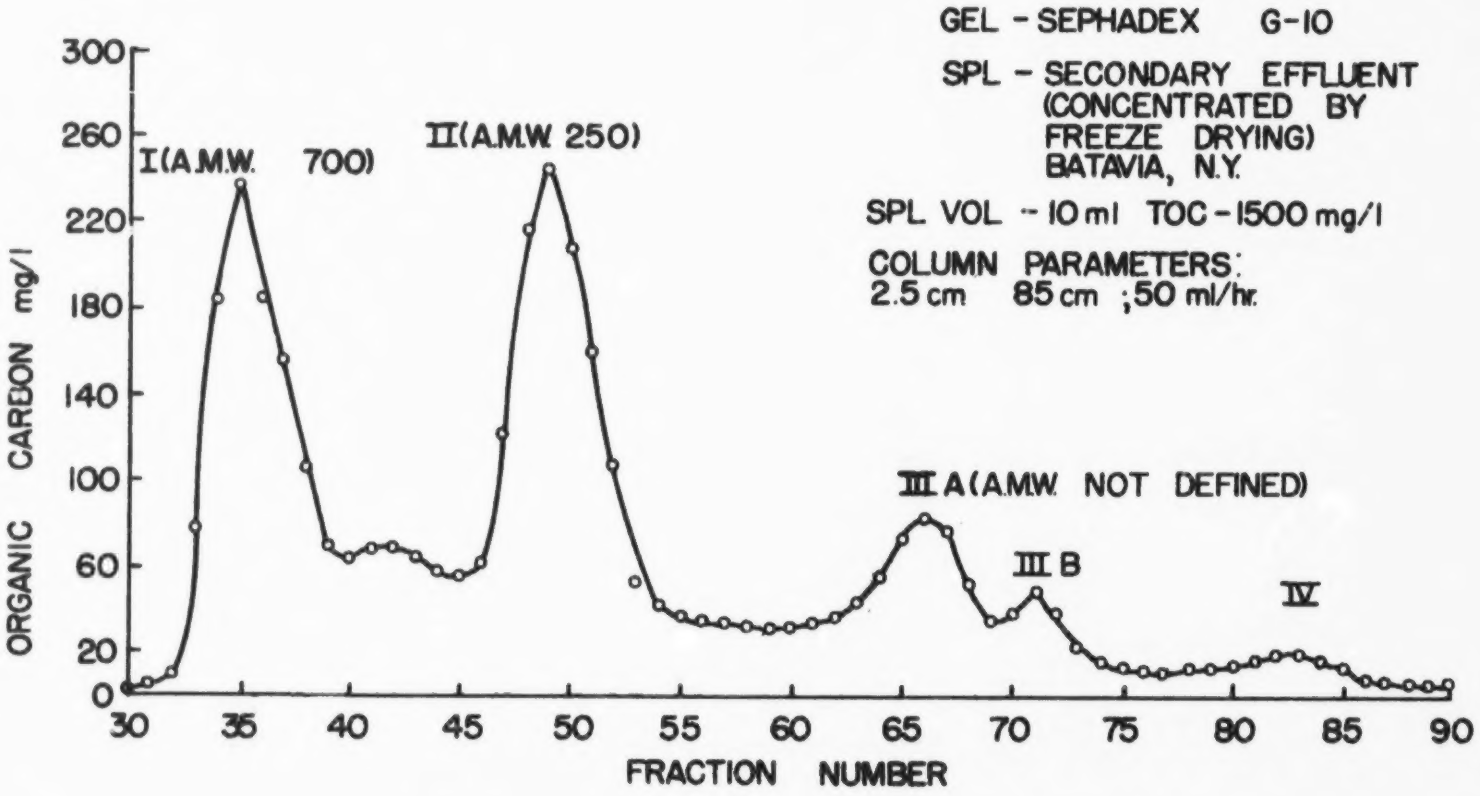


Fig. 13f ELUTION DIAGRAM OF THE FRACTIONATION OF CONCENTRATED BATAVIA EFFLUENT ON SEPHADEX G-10, RUN 6.

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Table 9. Organic Carbon Content of G-10 Fractions

Run	Organic Carbon Applied (mg)	Organic Carbon Content of Fractions (mg)				
		I	II	IIIa	IIIb	IV
1	15.0	4.3	4.1	1.4	0.66	0.38
2	15.0	4.3	5.6	1.5	0.73	0.43
3	15.0	4.5	5.4	1.6	0.64	0.27
4	15.0	5.1	5.6	1.7	0.63	0.32
5	15.0	4.9	5.2	1.8	0.77	0.33
6	15.0	4.8	5.6	1.7	0.65	0.36
Total	90.0	27.9	31.5	9.7	4.08	2.09

assure measurable carbon concentrations upon fractionation on the G-25 column.

This concentrate, containing 720 mg/l organic carbon, was applied to the G-25 column in two passes of 10 ml each. The resulting chromatographs are shown in Figure 14 as Runs 1 and 2. The frontal peak noted as G-25-I, commenced its appearance at fraction number 40, the same position noted for the initial appearance of the egg albumin standard during the column standardization procedure; and, the second peak denoted as G-25-II appeared in highest concentration at fraction number 80, very close to the calculated bed volume of the column. Using the G-25 standard curve apparent molecular weights of greater than 5,000 and 1,000 were assigned to peaks G-25-I and G-25-II respectively. The data in Table 10 show the total organic carbon content in milligrams of each of the G-25 fractions. Following the procedure used with the G-10 fractions, the determination of the percentage of original effluent organic carbon content was based upon these data.

Subsequent to the completion of fractionation, these G-25 fractions were stored in anticipation of further experimental procedures.

G-50 chromatographic data. The total organic carbon concentration of the composite G-25-I was found to be 64 mg/l. 129 ml of this fraction was then reconcentrated by freeze-drying and the final volume made up to 25 ml with glass distilled water. The total organic carbon concentration of this final volume was found to be 330 mg/l, indicating a recovery factor of 100%.

This G-25-I concentrate was then applied to the G-50 column in four passes. Although the column had been successfully standardized, the data developed from this particular set of chromatographic experiments showed some dissimilarity in the shape of the elution profile produced during each one of the passes. The G-50 column was then repacked and restandardized and the effluent from each of the four G-50 runs was composited. This composite was reconcentrated by freeze-drying and the final volume was made up to 10 ml. The total organic carbon concentration was found to be 380 mg/l.

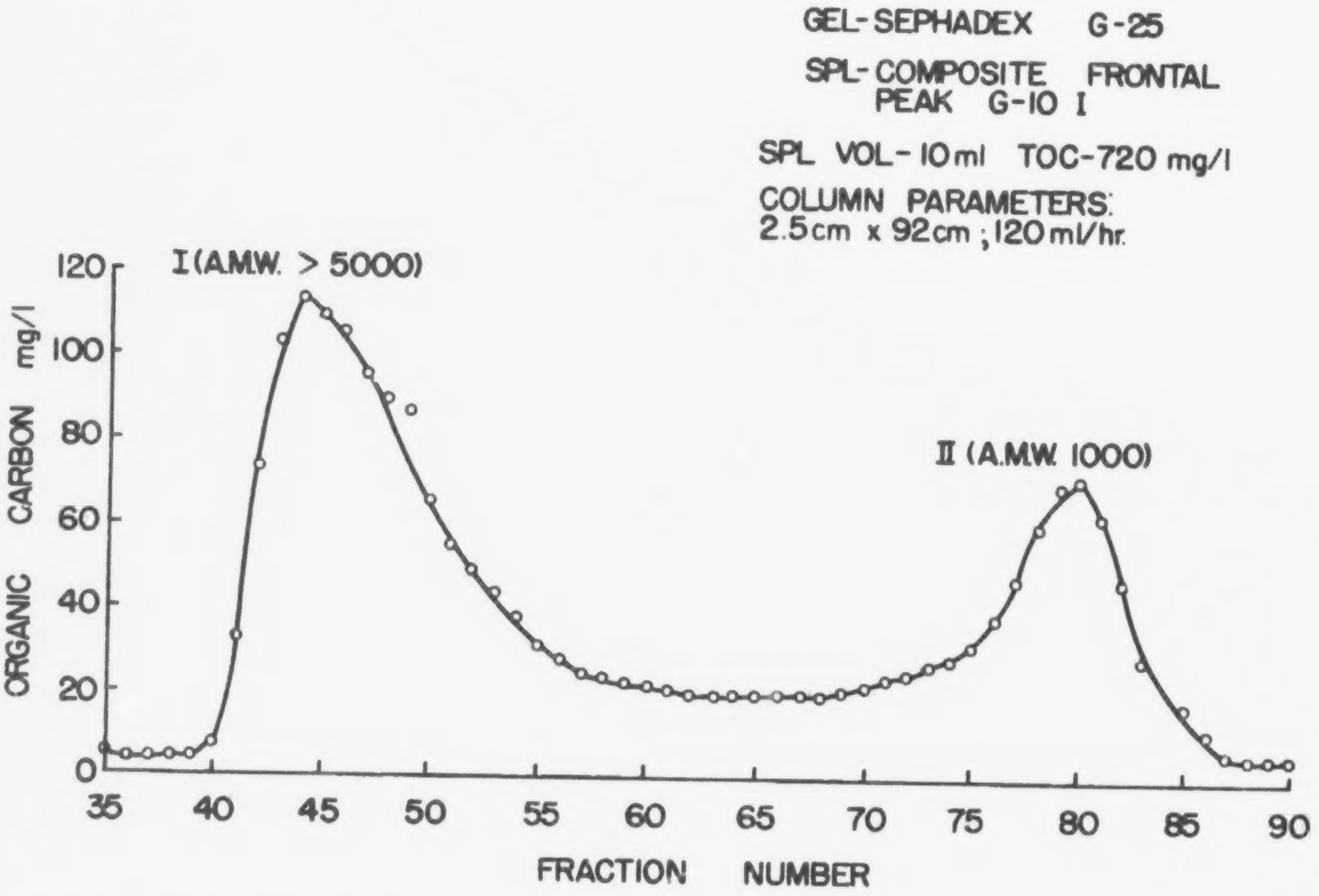

Fig. 14a Elution diagram resulting from the fractionation of concentrated composite frontal peak G-10-I on Sephadex G-25, Run 1.

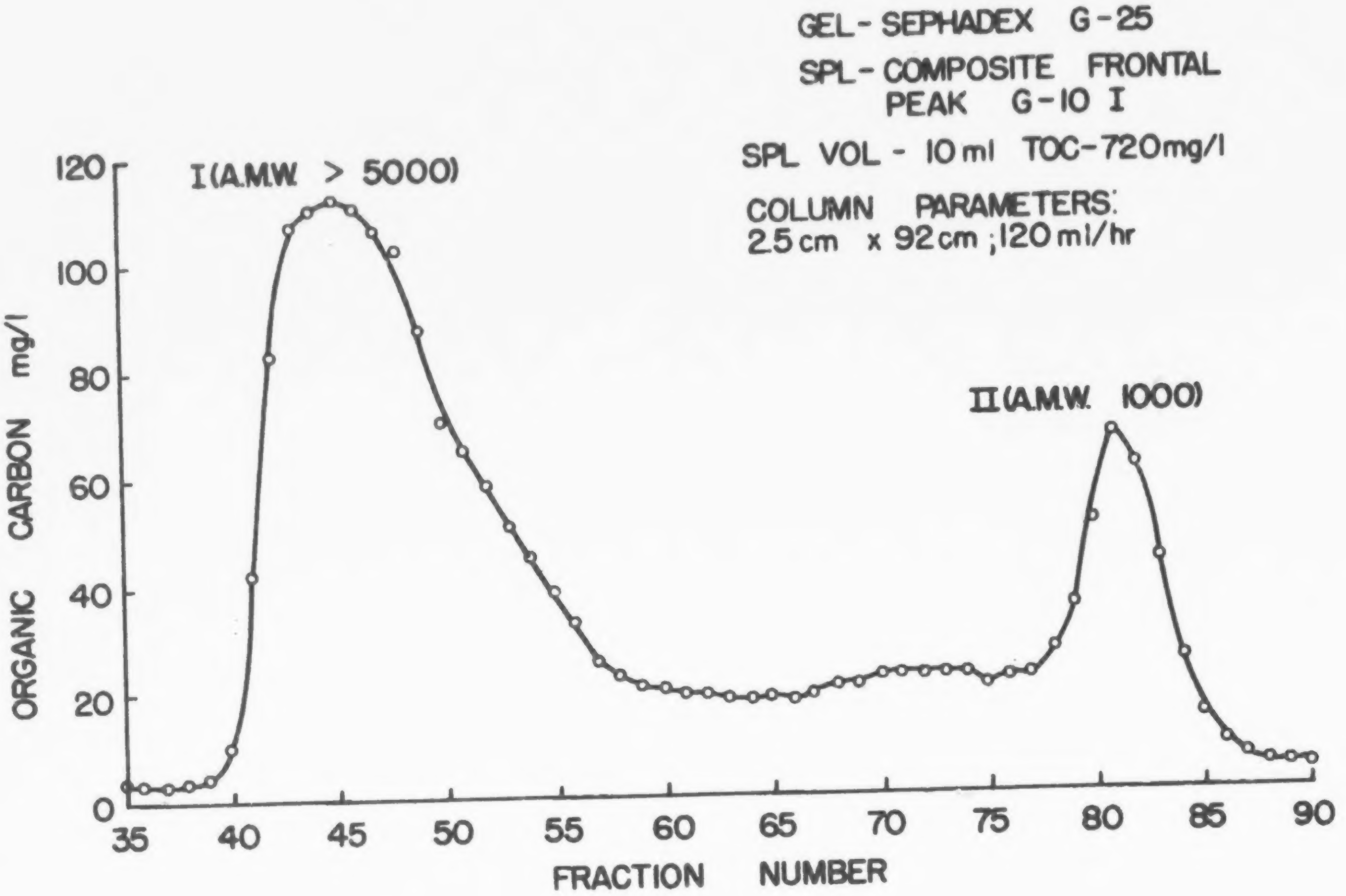

Fig. 14b ELUTION DIAGRAM RESULTING FROM THE FRACTIONATION OF CONCENTRATED COMPOSITE FRONTAL PEAK G-10-I ON SEPHADEX G-25, RUN 2.

Table 10. Organic Carbon Content of G-25 Fractions

Run	Organic Carbon Applied (mg)	Organic Carbon Content of Fractions (mg)	
		Front	II
1	7.2	4.9	1.9
2	7.2	5.5	1.5
Total	14.4	10.4	3.4

This secondary concentrate was then applied to the G-50 column in two passes of 4 ml and 5 ml each. The chromatograms produced upon fractionation of the G-50 column were similar and the peak in each case based on its point of appearance in the respective chromatograms adjudged to be a frontal peak and assigned a molecular weight of greater than 30,000. The G-50 chromatographs, produced as a result of these aforementioned final passes, are shown in Figure 15.

Table 11 depicts data regarding the total organic carbon content of the applied concentrate and the fraction produced by the G-50 column. As with the G-10 and G-25 data, these data were used to determine the percent of original effluent organic carbon content represented by the G-50-I fraction.

Table 11. Organic Carbon Content of G-50 Fractions

Run	Organic Carbon Applied (mg)	Organic Carbon Content of Fractions (mg)
		I
1	1.5	1.2
2	1.9	1.5
Total	3.4	2.7

Analyses of fractions. Data relative to the fractions obtained from the G-10, G-25 and G-50 fractionations are shown in Table 12. The data presented therein include total organic carbon concentration, pH, conductivity and apparent molecular weight. As evidenced from the conductivity data, the G-10 column succeeded in separating the great majority of the wastewater inorganic constituents from the frontal peak, the greatest percentage of these inorganic constituents appearing in fraction G-10-IIIa.

Conductivity determinations of the composite fractions were made to allow an interpretation of biological effects produced by the fractions in light of inorganic constituent concentrations. If the fraction did exhibit an effect, and its conductivity was low, then the interpretation of this effect as arising from an organic causative factor would be

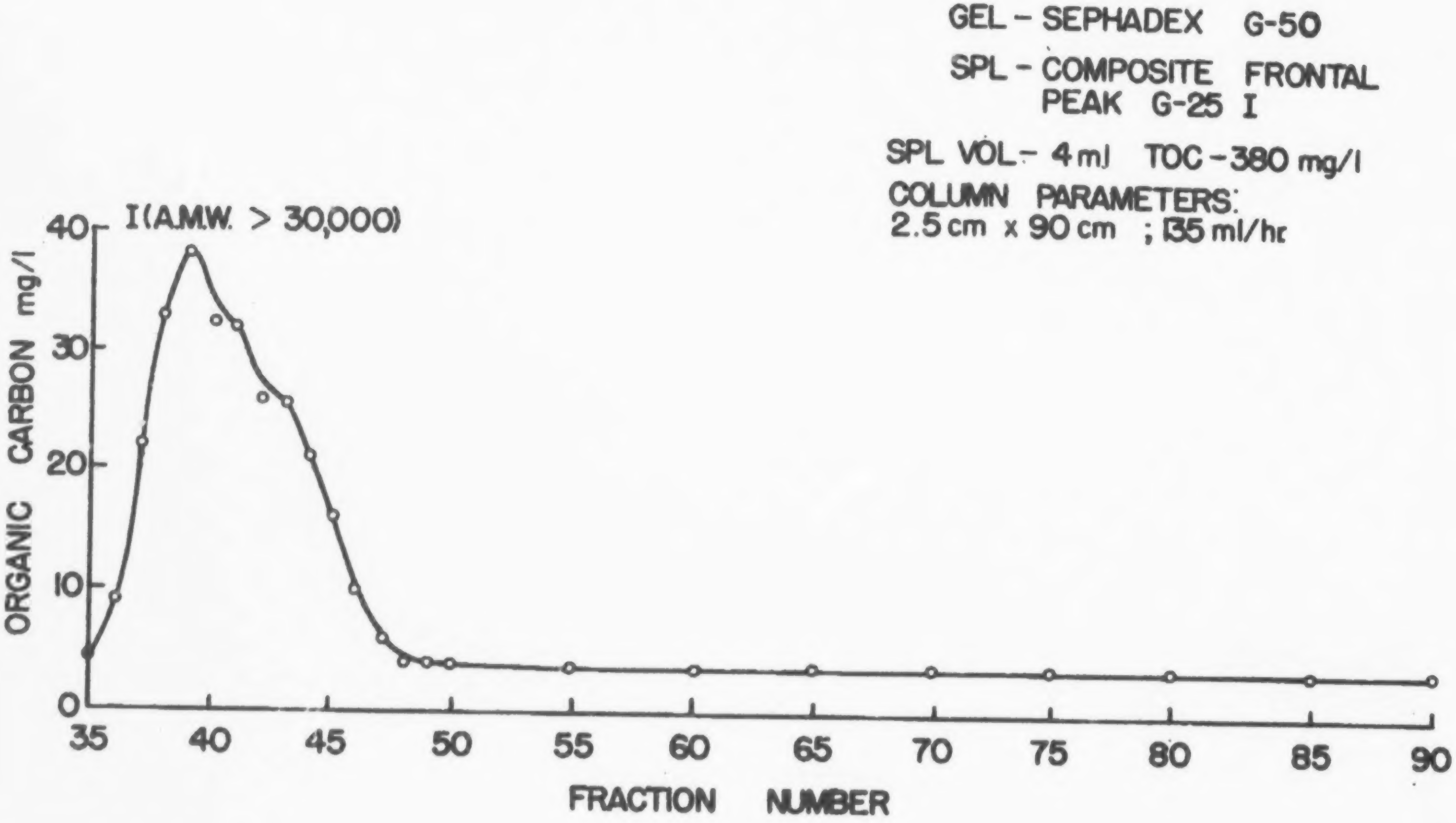

Fig. 15a Elution diagram resulting from the fractionation of concentrated composite frontal peak G-25 I on Sephadex G-50, Run I.

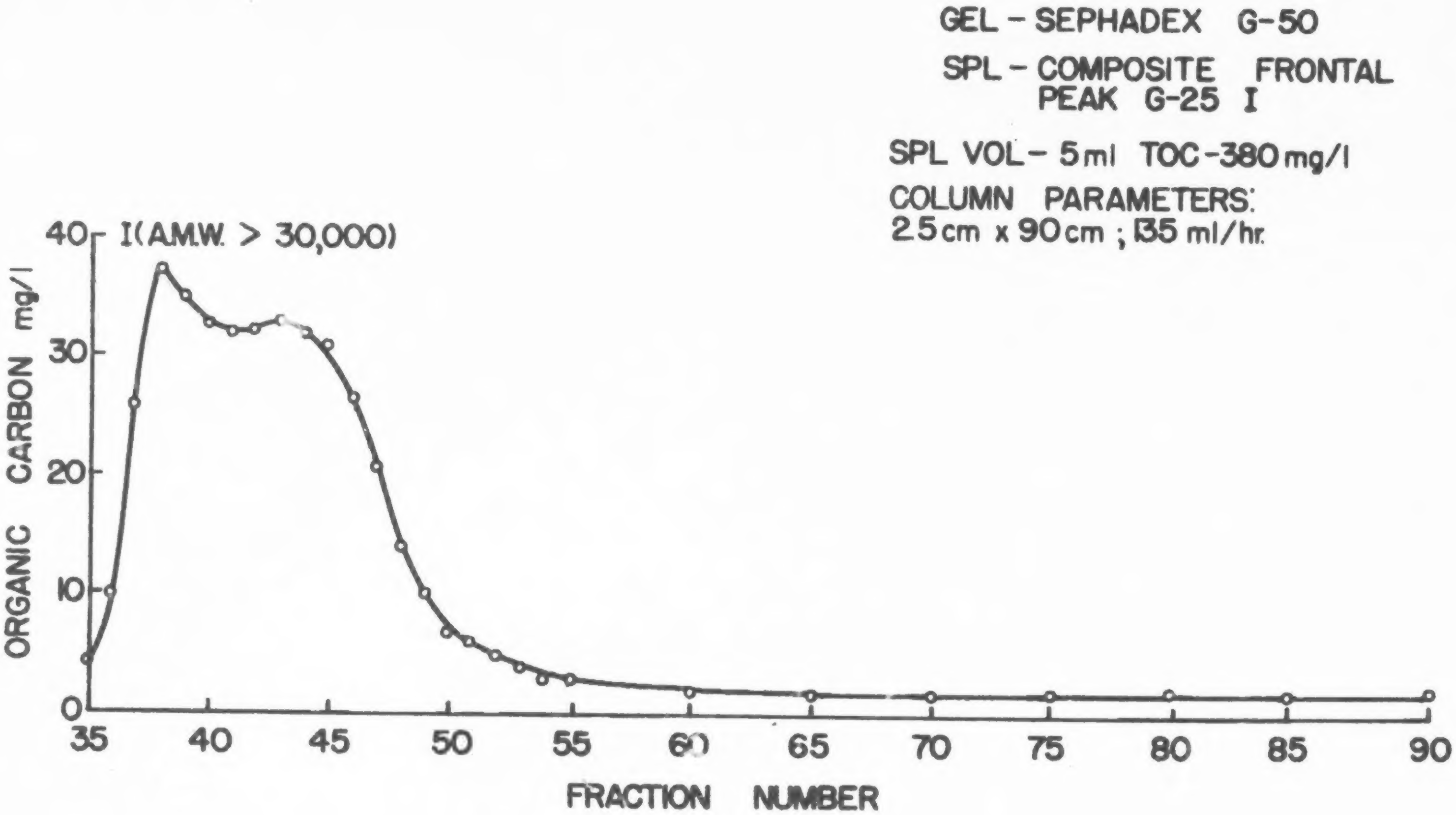

Fig. 15b Elution diagram resulting from the fractionation of concentrated composite frontal peak G-25 I on Sephadex G-50, Run 2.

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Table 12. Selected Parameters for Wastewater Fractions

Fraction	Total Organic Carbon Concentration mg/l	pH	Conductivity umhos/cm	Apparent Molecular Weight
G-10-I	120	5.6	850	>700
G-10-II	100	8.2	2,400	250
G-10 IIIa	60	7.1	20,000	*
G-10 IIIb	30	7.7	380	*
G-10-IV	17	6.3	34	*
G-25-I	64	7.1	56	>5,000
G-25-II	39	6.4	5.7	1,000
G-50-I	23	6.4	8.5	>30,000

* indefinable

appropriate. The organic carbon concentrations of the composite fractions were determined for use in the biological portion of the study. With these data in hand, and an approximation of the percentage of original effluent organic carbon content that each of the fractions represented, the volume of fraction required to be placed in a culture flask to match a set effluent dilution level would be easily obtainable.

Infrared analysis. Consideration was given to infrared analysis of the fractions as an aid in the identification of the organic compounds extant therein. One attempt was made to obtain the infrared spectrum of an organic fraction.

The fraction chosen for investigation was G-10-II. One ml of the chosen fraction was placed in a test tube, the tube fitted into a port on the freeze-drying apparatus, and the sample concentrated. Subsequently the dry sample was taken up with 10 ml of spectral quality chloroform and analyzed using a Beckman IR 20 spectrophotometer. No spectrum was obtained.

A generalization regarding infrared analysis requires that the unknown represent 10% by weight of the sample solution. Such a high percentage, even if only 1 ml of chloroform was used, would require more fraction volume than that obtained in the fractionations already performed, thus leaving no sample for the culture experiments. Based on the above, all further attempts at infrared analysis were deleted from the study.

Percentage of original effluent represented by fractions. As noted previously, calculations were performed to adduce the percentage of organic carbon applied to each column that the respective fractions represented. Using the postulate that concentration of freeze-drying and passage through a gel column allowed for no change in the basic character of the organic constituents in the concentrated effluent, one would then simply calculate the percentage of original effluent organic carbon content represented by such fractions. The above data are presented in Table 13.

In addition, this table also contains data relative to the organic carbon concentrations of each fraction in the original

Table 13. Percent of Original Effluent Organic Carbon Content in Fractions

Gel	Fraction	Percent of Organic Carbon Applied	Percent of Original Effluent Organic Carbon Content	Organic Carbon Concentration in Original Effluent (mg/l)
G-10	I	31	31	
	II	35	35	2.4
	IIIa	11	11	0.8
	IIIb	5	5	0.4
	IV	2	2	0.2
G-25	I	72	22	
	II	24	7	0.5
G-50	I	80	18	1.3

effluent. These data were based on an effluent carbon concentration of 7 mg/l. For example, as shown in Table 13, fraction G-10-II is shown to represent 35% of the original effluent organic carbon concentration. Thus, if such concentration was 7 mg/l, fraction G-10-II would be found in the original effluent to have a concentration of 2.4 mg/l.

Biological Investigations

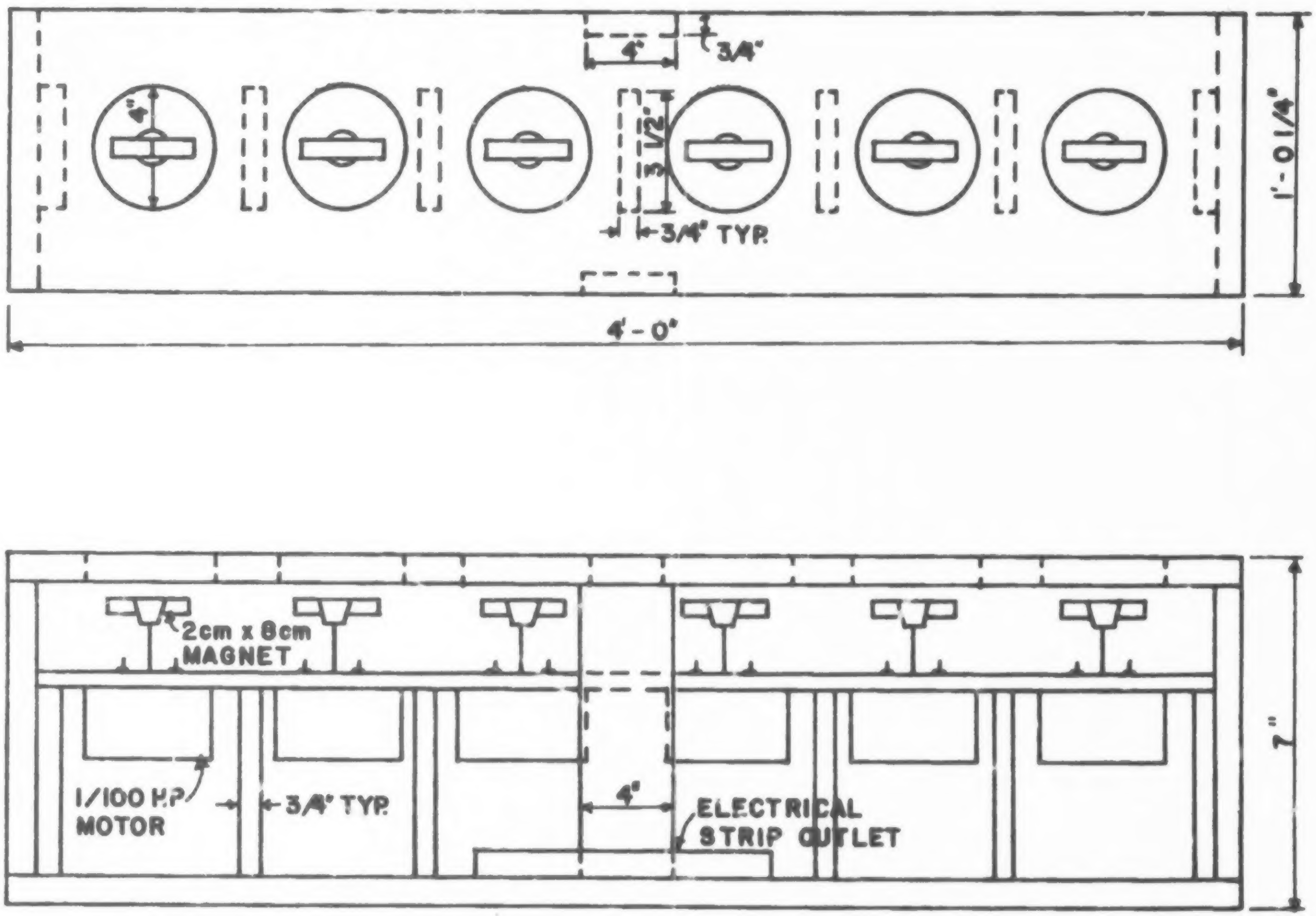
1. Materials and Procedures

To carry out the extensive biological investigations planned, it was decided that one room in the North Hall Laboratory would be converted into an algal culture room. To achieve this end, the windows of the room were covered with masonite to exclude extraneous light and a Carrier air conditioner installed to maintain the temperature of the room at 23 ± 1°C.

A continuously stirred batch culture apparatus noted in Figure 16 was constructed, using pressed board for the structure and 6 Dayton 1/100 horsepower motors for motive power. Six spots were provided on each one of the culture apparatus for the placement of algal culture flasks. A portion of a rubber stopper (size 7) was cut out to provide space for the placement of a 2 by 8 cm magnet. The magnet and stopper were then formed into a unit by wrapping the combination with ordinary uninsulated copper wire. The stopper was placed on the shaft of the motor so that as the shaft revolved, the magnet would also revolve. A teflon coated 4 cm magnet was put in each culture flask and the interaction of the magnets kept the cultures continuously stirred.

Sixteen of the continuously stirred batch culture apparatus were built, providing facilities for 96 individual algal culture experiments.

Suspended above the culture apparatus were banks of 40 watt fluorescent bulbs. These banks were suspended from bracing and assured that the irradiant setting for the algal culture experiments could, at will, be raised or lowered. The settings used in these experiments were 550-foot candles for *Selenastrum capricornutum* (85, 86) and 150-foot candles for *Anabaena flos-aquae* and *Microcystis aeruginosa*.


3/4"
4"
4"
3 1/2"
3/4" TYP.
1'-01/4"
4'-0"
2cm x 8cm MAGNET
1/100 H.P. MOTOR
3/4" TYP.
4"
ELECTRICAL STRIP OUTLET
7"

CULTURE APPARATUS

Fig. 16 CONTINUOUSLY STIRRED BATCH CULTURE APPARATUS.

The algae selected for use in this study were those proposed for use in the provisional algal assay procedure; Microcystis aeruginosa obtained from the laboratories of Dr. George P. Fitzgerald at the University of Wisconsin; and Selenastrum capricornutum and Anabaena flos-aquae obtained from the Pacific Northwest Laboratory, Federal Water Quality Administration, United States Department of Interior at Corvallis, Oregon.

The media used in the investigation were: 1/10 Gorham's medium, constituents and concentrations shown in Table 14 (45), and the basic ASM medium of the provisional algal assay procedure (47). The constituents and concentrations of basic ASM are as noted in Table 15. Basic ASM medium is the ASM medium of McLachlan and Gorham (60) with a reduction in the concentration of K_2HPO_4 from 17.4 mg/l to 3.48 mg/l, a reduction in the Na_2EDTA concentration from 7.4 mg/l to 1.0 mg/l and the addition of 50 mg/l of sodium carbonate.

Stock solutions of both media were made up as noted in the respective tables. Prior to a run of experiments, aliquots of the stock solutions sufficient to make up 18 liters of medium were discharged into 5 liters of glass distilled water and sufficient glass distilled water added to make up 18 liters. This bottle of medium was then stored in the cold room and used during the experimental procedure. The life of a bottle of medium at no time exceeded 3 weeks. Directly prior to experimentation, Erlenmeyer flasks containing aliquots of the medium were stoppered, autoclaved with the teflon coated magnet in place and stored in the culture room.

During the initial phases of the experimental procedures, only 1/10 Gorham's was used as the medium. It was discovered, however, that in using this medium reproducible growth rates for the cultures under investigation were not obtainable. This same effect was noted by Dr. Fitzgerald (29). It has been posited that the decrease in carbonate concentration seriously reduced the buffering capacity of the medium, thus allowing for wide and erratic pH changes which in the limit led to non-reproducible growth of the algae.

At this point, a switch was made to the basic ASM medium, in which the algae exhibited a reproducible growth rate subsequent to several inoculations.

Table 14. 1/10 Gorham's Medium

Compound	Stock Solution (g/l)	For 1 Liter of Solution Use (ml)	Final Concentration in Medium (mg/l)
$NaNO_3$	49.60	1	49.6
K_2HPO_4	3.90	1	3.9
$CaCl_2 \cdot 2H_2O$	3.60	1	3.6
Na_2CO_3	2.00	1	2.0
$MgSO_4 \cdot 7H_2O$	7.50	1	7.5
$NaSiO_3 \cdot 9H_2O$	5.80	1	5.8
Ferric Citrate	0.60	1	0.6
Citric Acid	0.60	1	0.6
$Na_2 \cdot$EDTA	0.10	1	0.1

Glass Distilled Water - Dilute to 1 Liter.

Table 15. Basic ASM Medium

Compound	Stock Solution (g/l)	For 1 Liter of Solution Use (ml)	Final Concentration in Medium (mg/l)
$NaNO_3$	8.50	10	85.0
K_2HPO_4	0.348	10	3.48
$CaCl_2 \cdot 2H_2O$	1.47	10	14.7
Na_2CO_3	5.00	10	50.0
$MgSO_4 \cdot 7H_2O$	4.90	10	49.0
$MgCl_2$	1.90	10	19.0
$FeCl_3$	0.032	10	0.32
$Na_2 \cdot$EDTA	0.100	10	1.0

Glass Distilled Water - Dilute to 1 Liter.

At the commencement of the investigation, the algae were cultured in 500 ml aliquots of the medium contained in 1 liter flasks. Subsequently it was determined that, to arrive at even a minimal concentration of the organic wastewater fractions in a 500 ml aliquot of medium, it would require inordinately large volumes of effluent fractions. Thus to reduce the volume of fractions required, the culture volume was reduced. At this juncture, the cultures were transferred and grown in 100 ml portions of Basic ASM medium contained in 250 ml Erlenmeyer flasks.

Upon transference to the new chosen conditions, the cultures did not immediately respond and several weeks passed before a representative and a reproducible growth rate could be obtained. *Microcystis aeruginosa* never recovered from the initial shock of the transfer. It was found that *Microcystis*, under the posited conditions, would only grow if the culture was left completely unstirred. A decision was made at this time that the study would be carried forward using *Anabaena flos-aquae* alone as a representative of blue-green algae and *Selenastrum capricornutum* as a representative of the green algae.

Throughout the investigation, the cultures were transferred at set times after initial inoculation. *Selenastrum capricornutum* being transferred from a culture flask that had been allowed to proliferate for 4 or 5 days and *Anabaena flos-aquae* from a flask growing for 6 to 7 days. The volume of inoculum used at all times was 1 ml. During the culture experimentation involving the organic fractions of the Batavia effluent, selected flasks were marked and the desired volume of organic fraction, based on previous calculations, was drawn up from the serum bottle into a syringe, and the contents of the syringe discharged directly into the culture flask. Immediately thereupon, the flask was inoculated along with controls and placed on the continuously stirred batch culture apparatus.

The growth of the cultures under investigation was followed, using spectrophotometric techniques. The chosen instrument was the Beckman DU-2 spectrophometer. The culture to be measured was treated as follows: a sample of the culture was removed from the culture flask with a 10 ml sterile disposable pipette and introduced into a 5 cm spectrophotometric cell, which had been previously acid-washed and set aside specifically for use with only one species of algae. The 5 cm cell was then placed in the DU-2 and the absorbance of the culture measured at 750 nm (94). After the measurement of the absorbance, the contents of the 5 cm cell were reintroduced into the culture flask and the flask returned to the continuously stirred batch culture apparatus.

Although absorbance measurements are widely used as reflections of the growth of a culture, cell counts are also performed in order to develop a correlation between the absorbance measured and the cell concentration of the sample. Selenastrum and Microcystis were counted with a brightline haemocytometer, using the red corpuscle method.

Since Anabaena flos-aquae is not a unicellular algae it did not lend itself readily to the cell counting method and thus a correlation between dry weight and absorbance was developed. After an absorbance had been measured 25 ml to 50 ml of the culture was removed from the flask and membrane filtered using a 0.45 u membrane filter and a 47 mm filter holder apparatus. Prior to the discharge of the culture onto the membrane filter, the filter was washed with glass distilled water. After completion of the filtration of the Anabaena flos-aquae, the filters were removed from the filter holder, placed in petri dishes and then in a constant temperature oven at 70^{o}C for 24 hours. Subsequent to this drying operation, the filters were allowed to cool for one hour and were weighed. The difference between this weight and original weight of the filter was interpreted as the dry weight of the algae. Dry weight measurements of a culture with a given absorbance were performed in triplicate and the individual weights in the series averaged after correction based on changes in the weight of a control filter. In both cases of cell count or dry weight determinations, sufficient observations were made so as to produce a statistically reliable correlation between the chosen parameter and absorbance.

For any given culture, whether spiked with a fraction or a control, a regression analysis was performed using those points determining the log phase of growth. Using this regression technique a growth rate was developed, which then allowed for a comparison of the growth characteristics of the cultures.

In addition, these investigations attempted to ascertain the effect of various concentrations of nitrogen and phosphorus on the growth rate of Selenastrum capricornutum. The media employed were as described in Tables 14 and 15 with one minor alteration in the modified Gorham's medium. The concentration of Na_2CO_3 was increased to 50 mg/l as an aid to pH control. Conditions of illumination and temperature were as noted previously.

For these experiments, the culture method employed bubbling tubes (ie, 25 x 200 mm optically matched pyrex test tubes filled with 25 mm of growth medium which was continuously aerated and mixed by passing water saturated air through a section of glass tubing that is vertically placed to the bottom of the culture tube). The growth of the culture was followed using a Bausch and Lomb Spectronic 20 at 750 nm and a one inch cell path.

2. Biological Results

Methodological considerations. Subsequent to the conclusion of the chromatographic portion of the study and the coincident garnering of the organic fractions, biological investigations were undertaken. As noted in the section on experimental procedure the test organisms to be studied, Selenastrum capricornutum and Anabaena flos-aquae, were cultured under the posited conditions both as controls and in culture vessels spiked with selected organic fractions or combinations thereof.

It was decided at the outset that the first sets of experiments would involve addition of the fractions in such amounts as to produce a final concentration of the selected fractions in the culture vessel approximating that as was found to exist in the filtered effluent (Table 13). Since it was felt that an effect not being had at this maximum level would in all probability not exist at a reduced concentration, adherence to this procedure would immediately allow one to note if and what fractions would produce a change in growth rate when added to the culture.

The response to the organisms to this fraction addition was, as noted previously, to be followed and ascertained by comparison and the growth rates developed with those found for the control cultures. The growth rate for an individual culture was determined by employment of a linear regression technique on those points of the growth curve representing the log phase of growth. For each set of experiments the individual growth rates found were then averaged to produce the mean growth rates presented herein.

To adjudge the validity of the mean growth rates obtained, such data were subjected to statistical analysis using Student's T distribution for small sampling theory. Based on this technique the 95% confidence interval for each mean growth rate was determined. These data then afforded a measure of significance of the difference in growth rates obtained with the controls and treated cultures.

Since the growth rates were to be determined by measurement of culture absorbance at 750 nm, it was decided that some correlation between absorbance at such wavelength and a chosen paramater of growth should be provided. Presented in Figure 17 are graphical presentations of such correlations. In the case of *Selenastrum capricornutum* this parameter was cell concentration and for *Anabaena flos-aquae* dry weight was determined in accordance with the method outlined in the experimental procedures section.

Effect of the organic fractions on the growth of Selenastrum capricornutum.

Maximum levels of organic fractions. Presented in Table 16 are data relative to the effect produced on *Selenastrum capricornutum* when exposed to the concentrated effluent and its organic fractions in the culture vessels. Noted in this table are the concentrations of the fractions and the growth rates found under the varying test conditions. Also shown therein are the 95% confidence intervals.

As can be seen from such data, three of the organic fractions, G-10-IIIb, G-25-II, and G-50-I, and the concentrated effluent caused significant responses in *Selenastrum capricornutum*. The largest changes in growth rate were produced with the addition of fraction G-50-I and the concentrated effluent, with minimal changes in growth rate demonstrated upon addition of the apparently low molecular weight fractions G-10-IIIb and G-25-II. The data also indicate the response found was selective in that no correlation between the increase in

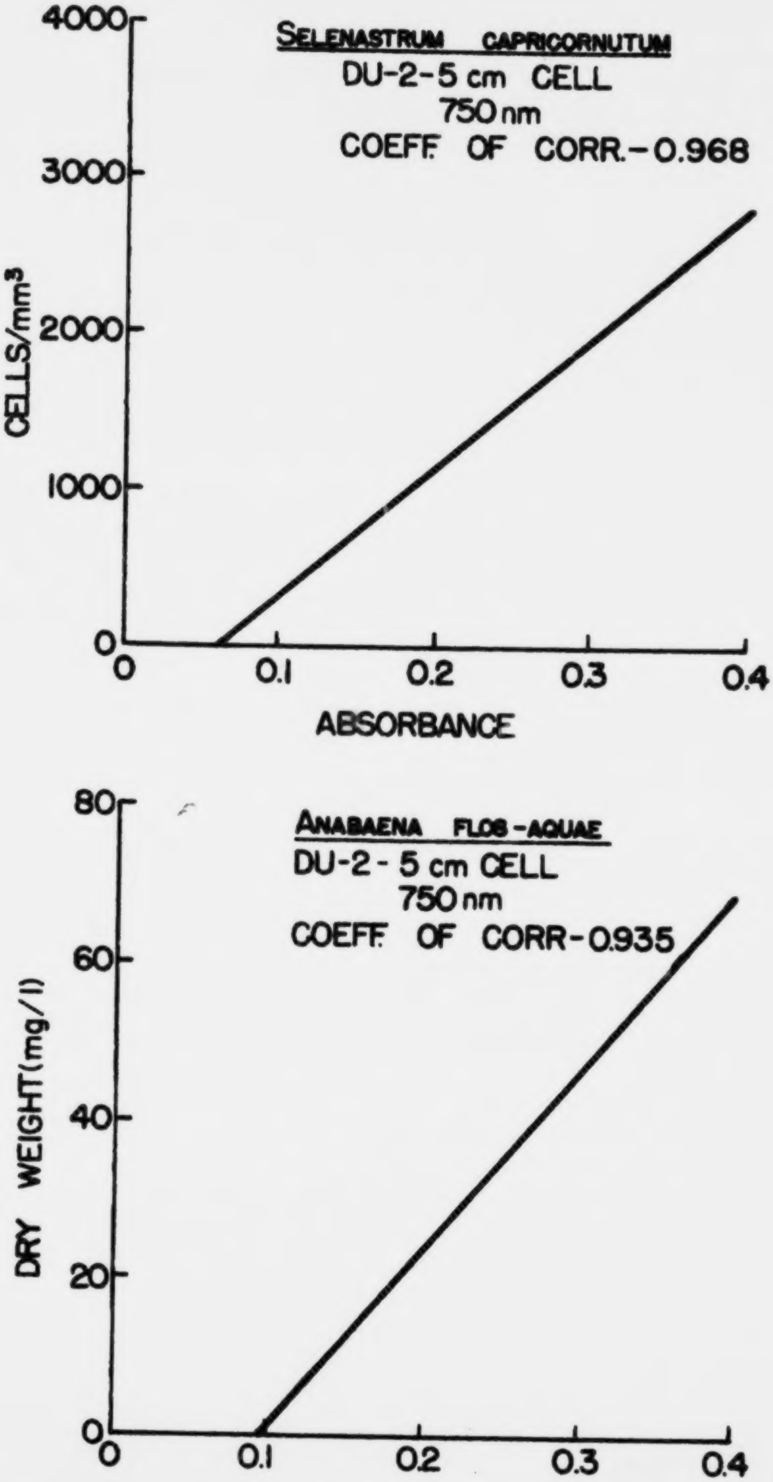


Fig. 17 GRAPHICAL REPRESENTATION OF THE CORRELATION BETWEEN ABSORBANCES AND SELECTED GROWTH PARAMETERS FOR *SELENASTRUM CAPRICORNUTUM* AND *ANABAENA FLOS-AQUAE.*

Table 16. Effect of Fractions on the Growth Rate of *Selenastrum capricornutum*

Fraction	Concentration of Fraction mg/l Organic Carbon	Growth Rate K_{10} (day^{-1})	Confidence Interval
Control	--------	0.43	0.42 - 0.44
G-10-II	2.0	0.43	0.41 - 0.45
G-10-IIIa	0.6	0.42	0.40 - 0.44
G-10-IIIb	0.3	0.49	0.48 - 0.50
G-10-IV	0.2	0.43	0.39 - 0.47
G-25-II	0.4	0.50	0.49 - 0.51
G-50-I	1.3	0.72	0.70 - 0.74
Concentrated Effluent	7.0	0.96	0.89 - 1.03

growth rate and the organic carbon concentration of the fractions in the culture flask is evident. In point of fact within the fraction series itself that fraction having the highest organic carbon concentration in the culture vessel produced no effect whatsoever.

Displayed in Figure 18 are growth curves one representative of the *Selenastrum capricornutum* control and the other of the culture with concentrated effluent added. These two curves represent the culture extremes encountered in the studies with *Selenastrum capricornutum*. Growth curves for the other fractions were found to lie in the area bounded by these curves.

Reduced levels of organic fraction. The data presented in the foregoing section represent the effect produced upon addition of the fractions and the concentrate at the postulated maximum concentration. It was decided that the effect of reduced concentrations should be investigated. The experiments in this section were narrowed so that only those fractions showing a growth enhancement at such maximum concentration were investigated. Table 17 contains data relative to the effect on growth rate when the organic fractions and the concentrate are present in the culture flask in reduced concentrations. In the case of fractions G-10-IIIb and G-25-II, the biostimulatory effect previously noted for these fractions is removed completely upon reduction to approximately 1/2 of the concentration originally eliciting a response.

In the case of fraction G-50-I and concentrated effluent, the initially high concentration used allowed for investigation of the effects of these entities at two reduced levels. The levels chosen were 1/2 and 1/10 of the initial concentration. As can be seen from the data, a reduction to the 1/2 level still afforded a growth rate higher than that found for the control. However, further reduction in their respective concentrations to levels 1/10 of the initial removed all vestiges of growth rate enhancement.

Figure 19 graphically represents the relationship between the growth rate attained versus the percentage of full complement organic carbon concentration for fraction G-50-I and the concentrated effluent.

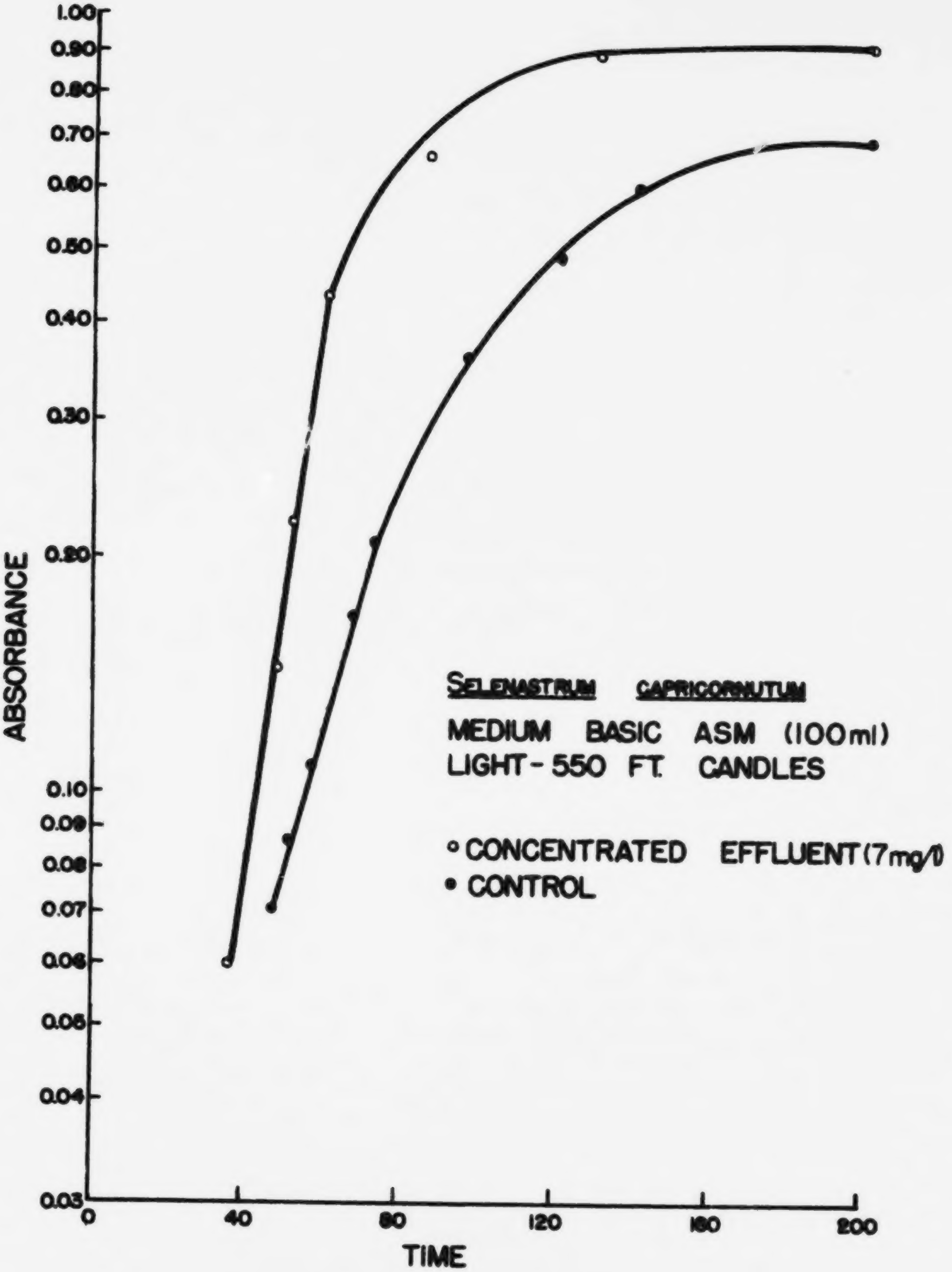


Fig. 18 SELENASTRUM CAPRICORNUTUM GROWTH CURVES.

Table 17. Effect of Organic Fractions in Reduced Concentrations on the Growth Rate of *Selenastrum capricornutum*

Fraction	Concentration of Fraction mg/l Organic Carbon	Growth Rate K_{10} (day^{-1})	95% Confidence Interval
G-10-IIIb	0.2	0.43	0.39 - 0.47
G-25-II	0.2	0.45	0.40 - 0.50
G-50-I	0.7	0.63	0.60 - 0.66
	0.1	0.43	0.40 - 0.46
Concentrate	3.5	0.86	0.79 - 0.93
	0.7	0.43	0.40 - 0.46

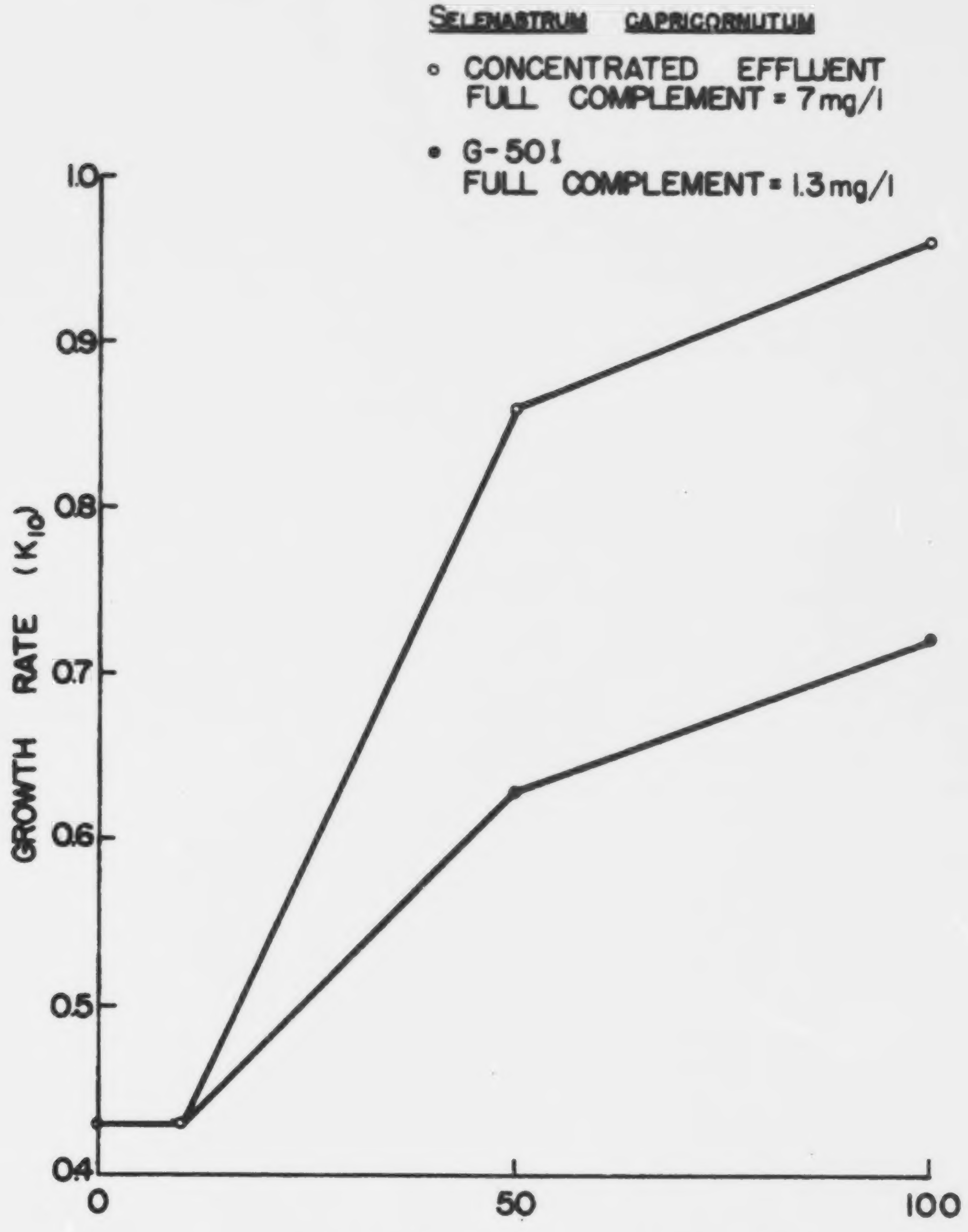


Fig. 19 GRAPHICAL REPRESENTATION OF THE RELATIONSHIP BETWEEN THE *SELENASTRUM CAPRICORNUTUM* GROWTH RATE AND VARYING ORGANIC CARBON CONCENTRATION OF THE CONCENTRATED EFFLUENT AND FRACTION G-50 I.

Effect of fraction combinations. Since the effects produced by fractions G-10-IIIb and G-25-II at the maximum concentration of 0.3 mg/l and 0.4 mg/l were small; and in that fraction G-50-I produced an effect not matching that of the concentrated effluent it was decided that combinations of the organic fractions should be tested to ascertain if and to what extent synergism played a part. The combinations chosen are as noted in Table 18.

The data displayed in Table 18 clearly show that the first combination used that of fraction G-10-IIIb plus G-25-II produced a growth rate equal to that had when the fractions were introduced individually into the culture vessel. It should be noted that in these combination experiments the concentrations of the individual fractions were as noted in Table 16.

The second combination involved fractions G-10-IIIb, G-25-II and G-50-I and the data from that run is also presented in Table 18. A synergistic effect seems to be evident in that the growth rate is substantially higher (0.85) than that produced by fraction G-50-I alone (0.72) and is very close to the range of the K_{10} produced by the concentrated effluent.

The effect of phosphorous was also investigated. In examining the organic fractions it was found that the highest concentration of total phosphorous existed in the concentrated effluent. The concentration extant in such concentrate was 1.5 mg/l as P while no discernible phosphorous concentration could be found in the several organic fractions. It was decided, therefore, to test the effect of the phosphorous contained in such concentrate on the Selenastrum capricornutum culture. In adding the volume of concentrate necessary to produce the maximum organic carbon concentration it was found that 3 micrograms of total phosphorous as P was added to the culture vessel under investigation. A sufficient volume of stock phosphorous solution was thus employed to add 3 micrograms to a set of Selenastrum capricornutum cultures. Such procedure involved the addition of 50 microliters of a 348 mg/l K_2HPO_4 solution. The K_{10} after addition of this minimal amount of phosphorous was discovered to be 0.50 ± 0.02, the same growth rate obtained with the addition of fractions G-10-IIIb and G-25-II individually or in combination.

Table 18. Selenastrum capricornutum Growth Rates Produced by Fraction Combinations

Fraction Combination	Growth Rate K_{10} (day^{-1})	95% Confidence Interval
G-10-IIIb + G-25-II	0.49	0.46 - 0.52
G-10-IIIb + G-25-II + G-50-I	0.85	0.82 - 0.88

Effect of the organic fractions on the growth of Anabaena flos-aquae. The same format followed in the experiments with Selenastrum capricornutum was used in the tests conducted with Anabaena flos-aquae. The sole difference, as noted in the previously set forth experimental procedure, involved a reduction in the level of illumination from 550 foot-candles to 150 foot-candles.

Maximum level of organic fractions. Outlined in Table 19 are the data relative to the effect of the organic fractions in the postulated maximum concentrations on the growth rate of Anabaena flos-aquae.

Those items eliciting a response from the organism were as displayed: G-50-I and the concentrated effluent. In contrast with the data developed during the experiments with Selenastrum capricornutum the low molecular weight fractions had no effect on the organism.

At first it was thought that perhaps an increased growth rate was being had when the fractions G-10-IIIa and G-25-II were used in the Anabaena cultures; however, a test of the significance of these data at the 95% confidence level indicated otherwise. As with the Selenastrum experiments, the concentrated effluent was discovered to have produced the most dramatic increase in growth rate with fraction G-50-I producing a lesser but strong effect.

It should be noted that the effects produced by these items in no way matched levels of increase determined with Selenastrum capricornutum, indicating that the sensitivity of Anabaena flos-aquae for the causative factors within the fraction G-50-I and the concentrated effluent was not as pronounced as that displayed by Selenastrum capricornutum.

Shown in Figure 20 are typical growth curves for an Anabaena flos-aquae culture both under control conditions and with the concentrated effluent added to the culture. These were the extreme conditions encountered in this set of experiments.

Reduced levels of organic fractions. Table 20 contains data regarding the growth rate responses experienced when fraction G-50-I and the concentrated effluent were applied at reduced levels to the cultures. The chosen reductions were as used previously 1/2 and 1/10 of maximum concentration.

Table 19. Effect of Organic Fractions on the Growth Rate of Anabaena flos-aquae

Fraction	Concentration of Fraction mg/l Organic Carbon	Mean Growth Rate K_{10} (day^{-1})	95% Confidence Interval
Control	-------	0.34	0.32 - 0.36
G-10-II	2.0	0.34	0.30 - 0.38
G-10-IIIa	0.6	0.38	0.34 - 0.42
G-10-IIIb	0.3	0.33	0.29 - 0.37
G-10-IV	0.2	0.34	0.29 - 0.39
G-25-II	0.4	0.37	0.33 - 0.41
G-50-I	1.3	0.41	0.38 - 0.44
Concentrated Effluent	7.0	0.54	0.50 - 0.58

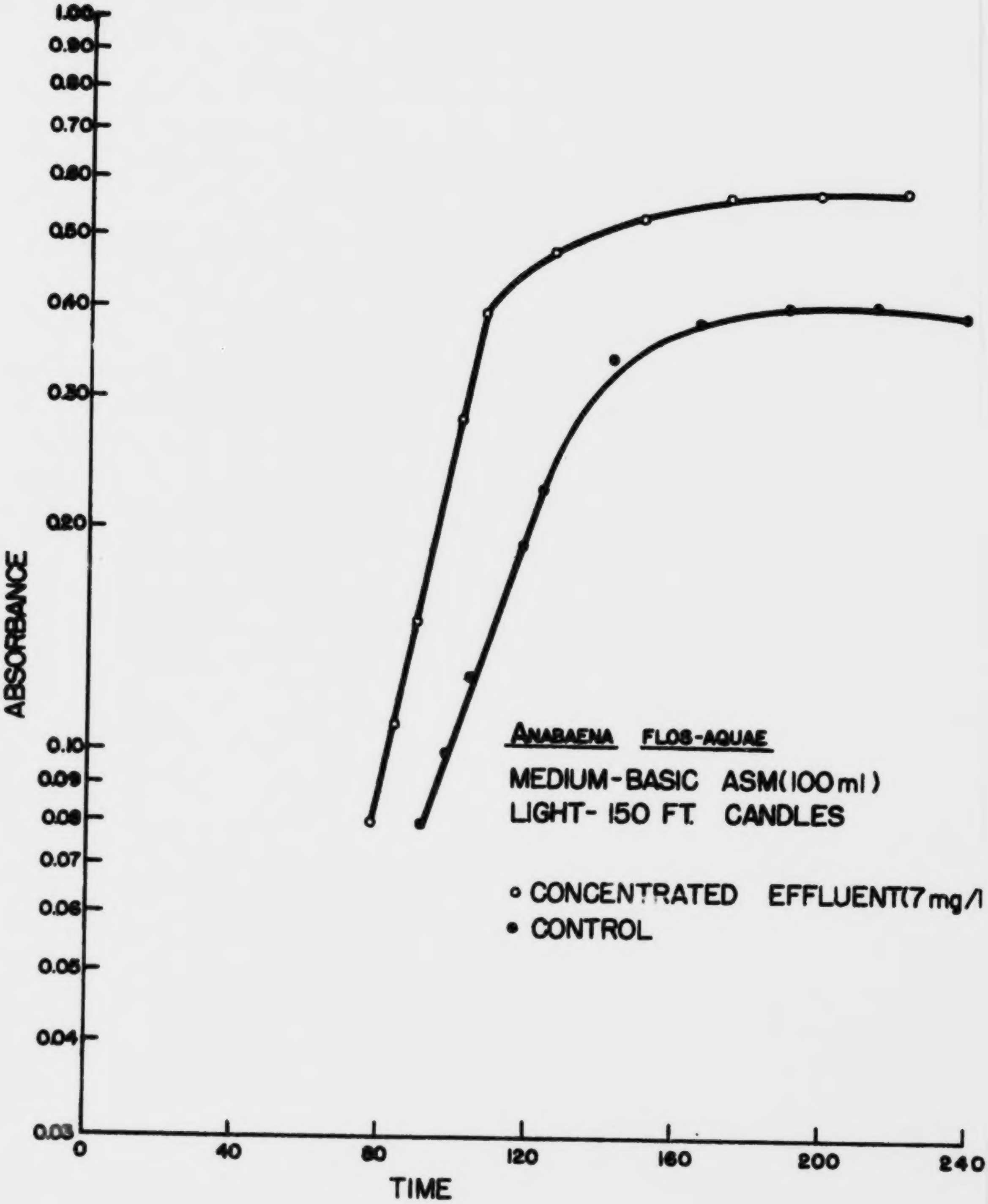


Fig. 20 Anabaena flos-aquae growth curves

Table 20. Effect of Organic Fractions in Reduced Concentrations on the Growth Rate of *Anabaena flos-aquae*

Fraction	Concentration of Fraction mg/l Organic Carbon	Mean Growth Rate K_{10} (day^{-1})	95% Confidence Interval
G-50-I	0.7	0.37	0.33 - 0.41
	0.1	0.33	0.30 - 0.36
Concentrated Effluent	3.5	0.41	0.39 - 0.43
	0.7	0.33	0.31 - 0.35

A reduction of 1/2 in the concentration of G-50-I extant in the culture flask removes all vestiges of growth rate enhancement. In opposition to this situation the concentrated effluent concentration had to be reduced to a level 1/10 of the original concentration before such occurrence would be had. At the 1/2 level the effluent had an effect equal to that produced by the G-50-I fraction in full complement. Figure 21 is a graphical representation of the relationship between K_{10} and the percentage of full complement organic carbon concentration for the concentrated effluent.

Effect of fraction combination. Since the data prior to statistical analysis indicated that G-10-IIIa and G-25-II could possibly have produced a response from *Anabaena flos-aquae* it was decided to investigate the effect of fraction combinations on *Anabaena flos-aquae*. The data so developed are as shown in Table 21.

Table 21. Anabaena flos-aquae Growth Rates Produced by Fraction Combination

Fraction Combination	Mean Growth Rate K_{10} (day^{-1})	95% Confidence Interval
G-10-IIIa + G-25-II + G-50-I	0.41	0.39 - 0.43
G-10-IIIa + G-25-II	0.36	0.34 - 0.38

The data presented clearly show that no interactive effects whatsoever were found. The K_{10} produced by the combination of G-10-IIIa, G-25-II and G-50-I was the same as that exhibited by the culture when G-50-I alone was introduced.

The result of the G-10-IIIa, G-25-II combination reinforces the findings ascertained with these fractions individually, namely, that they had no significant effect on the growth rate of *Anabaena flos-aquae*.

As was done during the *Selenastrum capricornutum* experiments, the effect of the phosphorous added with the concentrated effluent was also investigated. The growth rate observed in this experiment was 0.33 ± 0.03. In contrast with the effect produced by this minimal addition of phosphorous upon the

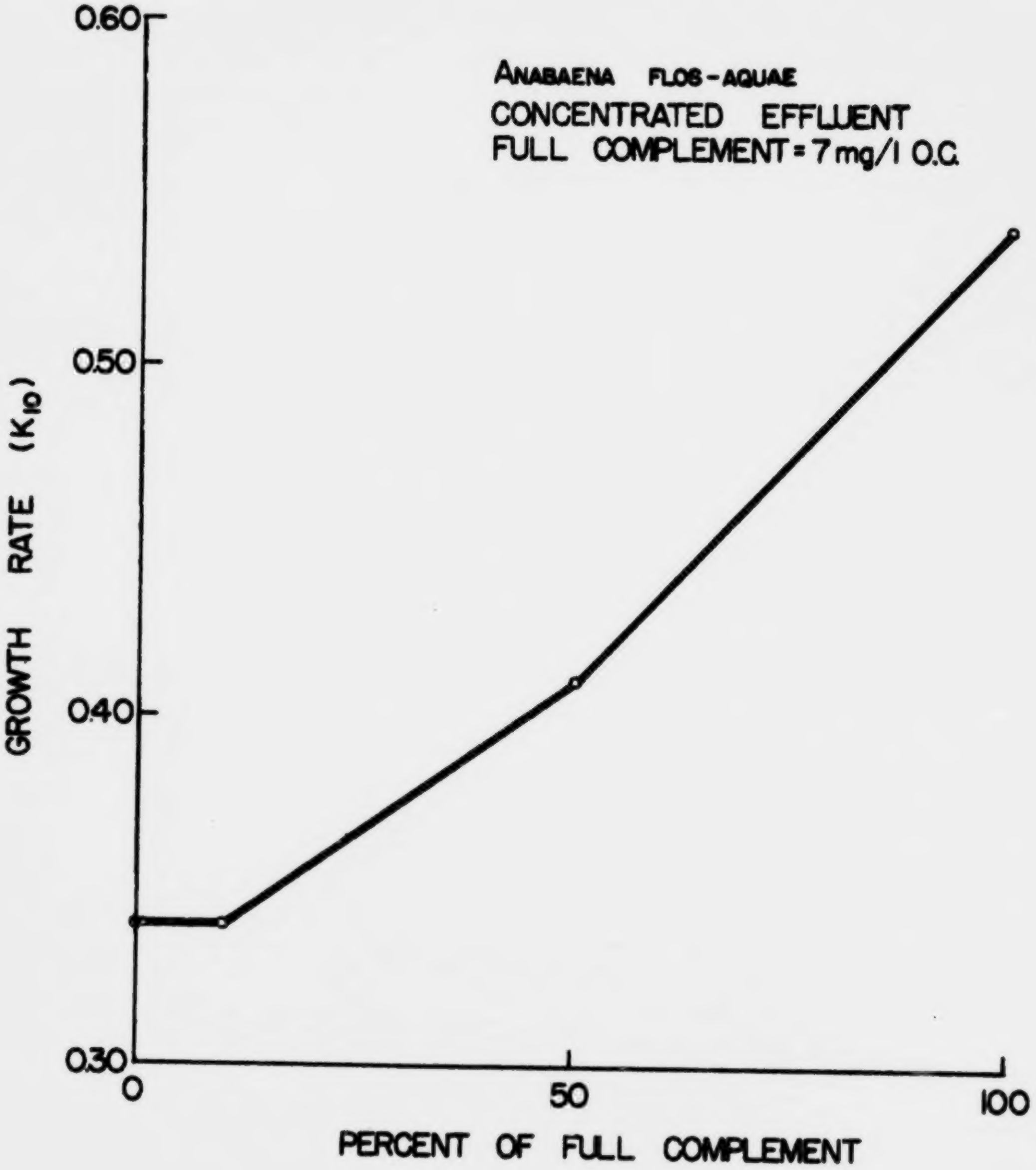


Fig. 21 GRAPHICAL REPRESENTATION OF THE RELATIONSHIP BETWEEN ANABAENA FLOS-AQUAE GROWTH RATE AND VARYING CARBON CONCENTRATION OF THE CONCENTRATED EFFLUENT.

growth rate of *Selenastrum capricornutum*, no effect whatsoever was adduced upon addition of phosphorous to the *Anabaena flos-aquae* culture.

SECTION V

DISCUSSION

Preliminary Methodology

The data developed during the initial phases of this study bear considerable note. Reference is specifically made to the use of freeze-drying as a concentration technique and the employment of gel chromatography to separate out into fractions the organic compounds extant in the test effluent.

Reviews of the literature for data relative to applications of the above techiques have elicited minimum evidence that such experimentations have been undertaken in the environmental fields. With reference to freeze-drying as a concentration method, the sole reference discovered is that of Painter and Viney (69) who employed freeze-drying in a study of the composition of domestic sewage. Gel chromatography experiments have been performed but most of the effort to date has centered around the fractionation of the organic material in natural waters, eg, the work of Gjessing and Lee (40). However, one report has recently been produced regarding the fractionation of raw wastewater and treated effluents using gel chromatography. This work was performed by Zunkerman and Molof in conjunction with their investigations of physical chemical treatment (97).

1. Freeze-drying

The results heretofore adduced, and more particularly noted in Table 6, clearly indicate that the freeze-drying technique was for these experiments superior to thin film rotary evaporation as a means of concentration. Although as noted previously the total percentage recovery was not obtained for thin film rotary evaporation, a comparison of the percentage recovery based on the soluble organic component shows that the freeze-drying technique afforded a substantial increase in percentage recovery for such component. When cognizance is taken of the possible problems encountered due to the effect of heat and coincident biological degradation on the sample to be concentrated, the freeze-drying technique again seems superior to any technique employing the addition of heat to the sample. Another point of interest lies in the lack of interaction among the many and varied components in the sample when freeze-drying is employed. This lack of interaction is due to the fact that the components within a sample are locked within the ice matrix and thus are not afforded an opportunity for interaction. Such is not the case with the thin film method.

Any question as to the efficacy of freeze-drying is dispelled upon examination of the data developed from the fractionation of the unconcentrated and concentrated water samples. The elution diagrams displayed in Figure 6 show that the technique produced a concentrate which is the equivalent of the original effluent sample and as a result selective losses of organic material were not evidenced.

When taken in concert, these four points namely, (a) the high degree of recovery obtained, (b) the minimization of any changes in the sample due to temperature effects, (c) the oviation of selective carbon losses, and (d) the lack of any interaction between sample components allow that this form of concentration is most effective. A strong, but secondary point in favor of this method, is the ease and simplicity with which the concentration may be carried out.

2. Gel Chromatography

It is indeed unfortunate that this process long the particular province of the biochemist has not been more extensively employed in the environmental field. It would seem that the technique would be most applicable to the characterization of wastewaters, both raw and as treated effluents. The data produced herein leads to some important considerations regarding the basic character of biologically treated wastewaters

Under the given conditions of analysis, it is evident that biologically treated wastewaters, whether they are effluents of activated sludge systems, trickling filters or extended aeration processes, contain organic compounds which are in the main of low molecular weight. The chromatographic data displayed hereinbefore show that 69% of the effluent organic carbon of the conventional activated sludge system at Batavia, New York was made up of organic compounds with an apparent molecular weight less than 700. Further analysis showed that 18% of the original sample organic carbon content has an apparent molecular weight in excess of 30,000. With respect to trickling filter and extended aeration effluents, it has been shown that the percentages of organic material with an apparent molecular weight less than 700 are 72 and 71 respectively.

The above results coincide with those arrived at by other investigators in their studies of the composition of wastewaters. For example, Dean et al (19) have found through their experiments that the low molecular weight fractions represented approximately 70% of the organic carbon. In another study, Bunch et al (11) indicated that low molecular weight compounds make up 60% of the effluent organic carbon. However, data not consonant with the above has been presented by Zuckerman and Molof (97). They employed gel chromatography as a means of assessing the validity of their proposed hydrolysis reaction in the physical chemical treatment scheme. Based upon their data, they have averred that the effluent from an activated sludge system would only contain organic compounds with a molecular weight greater than 1200. This contention does not seem to be correct in light of the data developed herein. Their results it should be noted were developed from analysis of an unconcentrated sample with the chromatographic effluent continuously monitored for COD.

Growth Rate Response of the Algae

Significant effects on the growth rate of the test organisms under investigation were evident upon addition of certain fractions and the concentrated effluent to the culture vessels. As noted previously growth rate enhancement was discovered upon addition of fractions G-10-IIIb, G-25-II and G-50-I and the concentrated effluent to the *Selenastrum capricornutum* cultures. Enhancement was also noted when the *Anabaena flos-aquae* cultures were spiked with fraction G-50-I and the concentrated effluent.

A review of the data in Table 12 allows for a preliminary assessment of the causative agency for such effects. Noted in such table are data relative to the conductivity of each of the chromatographic fractions. The conductivity of the active fractions ranged from a low of 5.7 umhos/cm for fraction G-25-II to a high of 380 for fraction G-10-IIIb with the high molecular weight fraction G-50-I having a conductivity of 8.5. In light of this information it could definitely be assumed that any effects being had with fractions G-25-II and G-50-I were due solely to an organic agency. In the case of fraction G-10-IIIb such a clearcut determination is not possible. The high conductivity evidenced by this particular fraction may be an indication that the increased growth rate is the result of inorganic factors or possibly organic ions. One possible explanation for such an effect could be the provision of a trace element or elements unavailable from the Basic ASM medium.

The results generated by addition of the fractions both singly and in combination bear note. Fraction G-50-I in the case of Selenastrum capricornutum produced the most dramatic and wide ranging effects. As can be seen from Figure 19 the effect produced by this fraction closely paralleled that found with use of the concentrated effluent. In point of fact the combination of G-50-I with G-25-II and G-10-IIIb produced a growth rate almost equal to that developed by the concentrated effluent. Based on these data it would not be amiss to assume that the major portion of the growth rate enhancement found upon testing of the concentrated effluent was due in the main to the action of the several fractions. Anabaena flos-aquae was in no way similarly affected. Response was developed only with the concentrated effluent and fraction G-50-I and the effects were not as strong as those exhibited with the Selenastrum capricornutum culture. The percentage increase in growth rate was much smaller for Anabaena for both fraction G-50-I and the concentrations of fraction G-50-I.

The concentrations of the fractions used in this study is a point of interest. Reference is specifically made to the use of zero dilution of the effluent as an upper limit. Such a selection, it is felt, is perfectly acceptable in light of the existence of wastewater treatment facilities on streams and water resources incapable of providing more than minimum dilution of the effluent. It would seem that the data developed indicate a pressing need for the reassessment of siting procedures, and treatment method selection. The need for such reassessment is reinforced when one considers that growth rate response was evident upon usage of reduced concentrations of fraction G-50-I and the concentrated effluent. The present tack taken by the regulatory agencies it is felt will lead in the near future to a mandate for the removal of phosphorus in treatment facilities, as phosphorus has been popularly indicated as the prime causative agent for increased algal growth in the aquatic environment. Based on the data herein presented, however, it would seem possible that in certain situations serious algal problems would remain notwithstanding the removal of phosphorus from the effluent.

Recent work conducted at Lake Tahoe by Middlebrooks, et al (63, 64) has indicated, as have previous investigations, that the biostimulatory effects of secondary effluents are considerable. The investigations conducted closely paralleled the techniques followed in this study. The main points of comparison are the employment of the batch culture technique, the use of growth rate as an indication of response and the use of an organism of the genera Selenastrum, namely, Selenastrum gracile. The experiments undertaken showed that the addition of secondary effluent to Lake Tahoe water produced marked enhancement of the growth rate of the organism. In addition, it was noted that the effects could in part be the result of the addition of biostimulatory agents in the effluent such as organic material, vitamin B_{12}, etc, as the growth rate responses were in some cases higher than could be accounted for solely on the basis of the amount of nitrogen and phosphorus in the sample. At this juncture it would be well to discuss the nature of the factors effecting these results.

1. Carbon

The first point to be discussed is the relationship of this enhancement of growth rate to the concentration of carbon in the fractions and concentrated effluent. As shown in Tables 16 and 19 the full fraction concentration expressed as organic carbon ranged from a low of 0.2 mg/l for G-10-IV to a high of 2.0 mg/l for G-10-II. It could be assumed that the effect produced upon addition of the stimulatory fractions was due to bacterial degradation of the organic carbon, the release of such carbon into the system and its subsequent use by the algal species.

However, to accept such a premise requires that several important assumptions be made. The first of these being that Basic ASM medium is carbon limited, which is definitely not the case. The second relates to the biodegradability of organic compounds. For example, one would have to assume that organic compounds of molecular weight greater than 30,000 were more subject to biodegradation than those existing in fractions G-10-II which had an apparent molecular weight of 250. Such an assumption it is posited is not supportable. Also if one was to assume equal opportunities for bacterial attack then one would be forced to accept the proposition that approximately equal concentrations of carbon qua carbon, 2.0 mg/l for fraction G-10-II versus 1.3 mg/l for fraction G-50-I had produced vastly different growth rate responses.

2. Phosphorus and Nitrogen

The cursory investigation undertaken in this research with phosphorus clearly indicates that if at all phosphorus played a minor role in the increased growth rate response of the organisms. As far as the individual fractions are concerned phosphorus could have played no part whatsoever since when tested for total phosphate no measurable concentration was discovered in any of these fractions.

In the case of the concentrated effluent it was of course noted that the addition of phosphorus in the same amount as would be added by this concentrate afforded an increase in the growth rate of Selenastrum capricornutum. This increase growth rate, 0.50, however, does not approach that produced by fraction G-50-I, 0.72, and is minimal when compared with that produced by the concentrated effluent, 0.96. Anabaena flos-aquae was totally unaffected by the addition of phosphorus.

In the case of nitrogen the analysis is not as straight forward as that noted previously for phosphorus. Investigation of the nitrogen content of the chromatographic fractions proved to be impossible due to the small amounts of such fractions available for all experiments; thus, the part played by nitrogen must be examined indirectly.

In the case of the high molecular weight fraction G-50-I, data developed in previous investigations (59) indicate that the achievement of a growth rate response equal to that produced by this fraction (0.72) would require the addition of 0.6 mg of nitrogen to the culture. Such an amount of nitrogen could not be made available from the organic compounds contained within fraction G-50-I. The conductivity exhibited by this fraction would immediately remove any consideration of an inorganic nitrogen source. Thus it would seem that the increased growth rate response caused by fraction G-50-I must be the result of a factor other than nitrogen.

The situation regarding the part played by nitrogen in the effects exhibited by fractions G-25-II and G-10-IIIb is unclear. Fraction G-10-IIIb possessed a conductivity of such magnitude as not to allow one to discount the possibility that inorganic nitrogen compounds in such fraction were responsible for the increased growth rate of *Selenastrum capricornutum*. In addition the organic compounds extant in the fraction may also have served as a source of nitrogen for the organism. The low conductivity of fraction G-25-II does not permit an interpretation of its effects as having been produced as a result of the presence of an inorganic nitrogenous compound in said fraction. However, the same may not be said for an organic nitrogen factor.

Since *Anabaena flos-aquae* was solely affected by fraction G-50-I it would seem that the general import of the discussion noted previously would hold. In addition since it has been noted that this organism is capable of nitrogen fixation it is surmised that its susceptibility to variations in nitrogen concentration would not be as marked as that shown by *Selenastrum capricornutum*.

3. Other Possible Causative Factors

Several possible alternative explanations may also be advanced for the effects exhibited in this study. First among these would be an accounting for such effects based on the provision of a requisite vitamin or a growth substance such as 3-indoleacetic acid. Both of these items it has been pointed out by Painter and Viney (69) have been discovered to be constituents of wastewaters. It would seem, however, on the basis of molecular weight considerations that the only such substance with a match to the fractions obtained would be vitamin B_{12}. The apparent molecular weight of fraction G-25-II would present a range for the presence of said substance. However, it should be noted that a vitamin requirement has yet to be established for *Selenastrum capricornutum*.

A possible explanation with application to all the active fractions would be chelation; but an assumption such as this would require one to discount the effectiveness of the $Na_2 \cdot EDTA$ already present in the Basic ASM medium.

A second consideration involves a parallel to that explanation proposed by Prakash and Rashid (73) for the effect of humic substances on marine algal growth. This explanation supposes that a factor or factors within the fractions act as sensitizing agents for cellular transport. It is surmised that such agents act to enhance the permeability of the algal cell membrane and thus increase the uptake of required nutrients from the basal medium.

Final consideration may be given to the possible addition of trace metals in the active fractions as biostimulatory agents. However, it would seem in light of the extremely low conductivities exhibited by fractions G-25-II and G-50-I, that only fraction G-10-IIIb would be capable of assuming this role. In addition it would not be amiss to assume that the sum total of the trace element requirements for both algal species had been satisfied by trace element impurities in the stock chemicals used to make up the basal medium.

4. Summary

The main thrust of the study hereinbefore delineated has been the garnering of organic wastewater fractions and the ascertainment of their effect on algal growth. This work has substantially been based on kenetic considerations. A fruitful field of investigation would be the assessment of ultimate growth effects. Such investigation would be of possible assistance in offering an explanation of the effects noted herein. The explanations now proffered for such effects are suppositive in nature. The true assessment of the causative factors involved must await an expansion of the conventional wisdom.

As mentioned, the effect of various concentrations of phosphorus and nitrogen on the growth rate of *Selenastrum capricornutum* was also investigated. The experimentation consisted in obtaining growth rates of the test alga in Basic ASM and modified tenth Gorham's media, both of which used glass distilled water and/or 0.45 u membrane-filtered Lake George water (an oligotrophic soft water lake in the eastern Adirondack Mountains) as dilution water. The rationale for using glass distilled water and Lake George water was to allow interpretation of the adequacy of the synthetic media for algal culturing. Once these growth rates were established, the concentrations of nitrogen and phosphorus in the two media were fractionally reduced to one-half and one-quarter of the full amount and the growth rate at each concentration level ascertained.

For the following illustrations, the ordinate indicates the extent of growth attained in 24 hours on a relative basis with Basic ASM medium made up with glass distilled water the norm for comparison since this medium supported the greatest rate of growth. During the experimentation only the component under study was altered from its concentration in the original medium. As illustrated in Figure 22, the effect of nitrogen reductions on the growth rate is less severe than fractional reductions in the phosphorus concentrations. The growth rate for _Selenastrum_ _capricornutum_ in Basic ASM is 1.32. In noting the effect of nitrogen reduction, it can be seen that a 50% reduction in nitrogen concentration produced a growth rate of 0.96 or a one-third reduction in cell concentration over the 24 hour period. This is contrasted with a reduction in K rate to 0.74 when the nitrogen is further reduced from 0.5 nitrogen to 0.25 nitrogen. The overall change in cell concentration produced by a decrease in nitrogen concentration from the full nitrogen level to one-fourth was 40%.

The influence of changes in phosphorus concentration is also illustrated in Figure 22. A reduction in cell concentration of 40% was affected by a 50% reduction in phosphorus level. A further reduction in the phosphorus level to one-fourth of the full medium phosphorus level produced an overall reduction in cell concentration of 45%. The experiments indicated in Figure 22 and the following figures are illustrative of the nutrient limitation exhibited by the decreased concentrations of the required elements, nitrogen and phosphorus, with the organism being consistently more sensitive to reductions in phosphorus concentration than nitrogen concentration.

In Figure 23 the data were obtained by using the Basic ASM medium with Lake George water substituted for glass distilled water. The striking result evident in these data is the

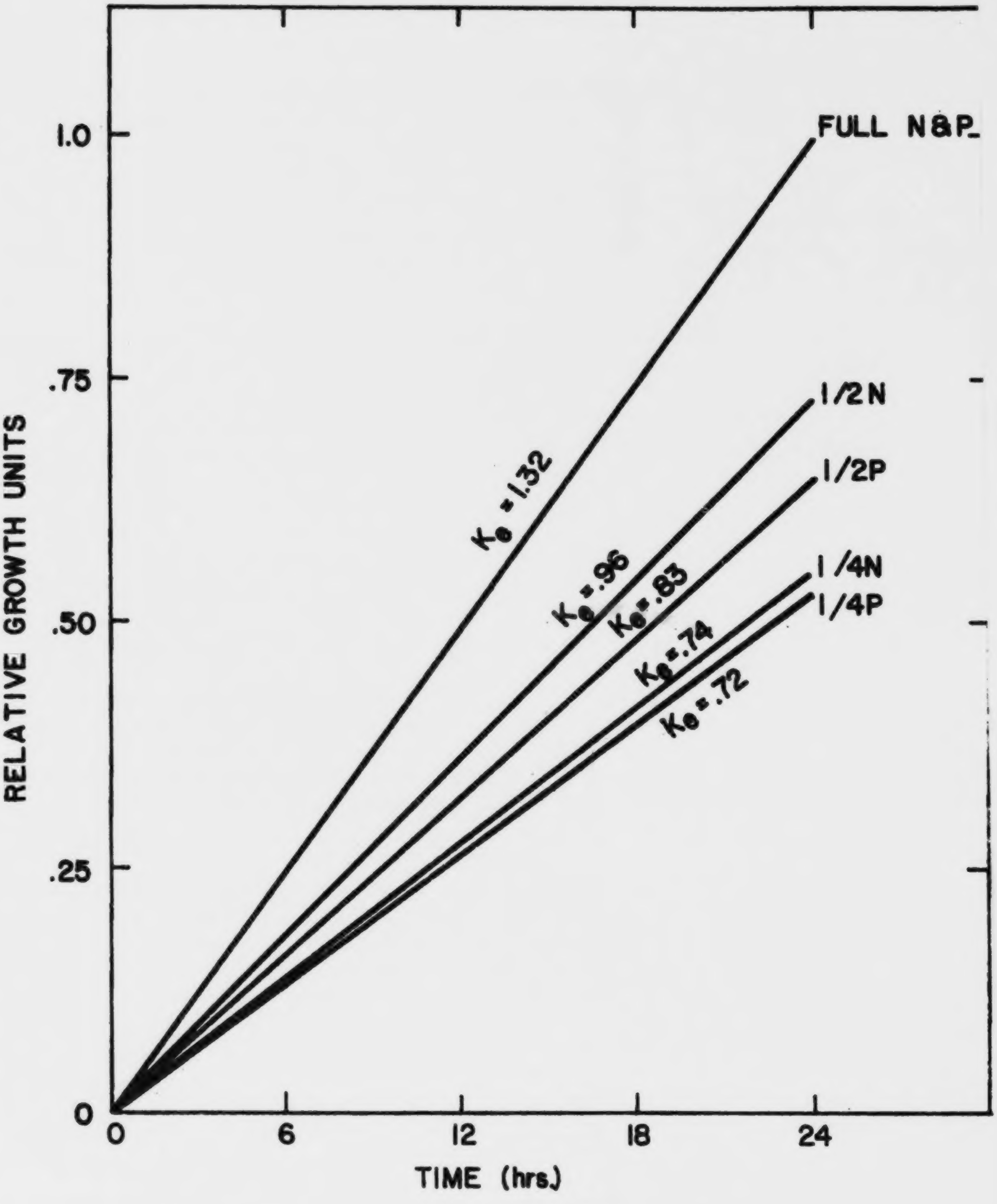


Fig. 22 EFFECT OF VARYING NUTRIENT CONCENTRATION IN ASM (DD) FOR SELENASTRUM CAPRICORNUTUM.

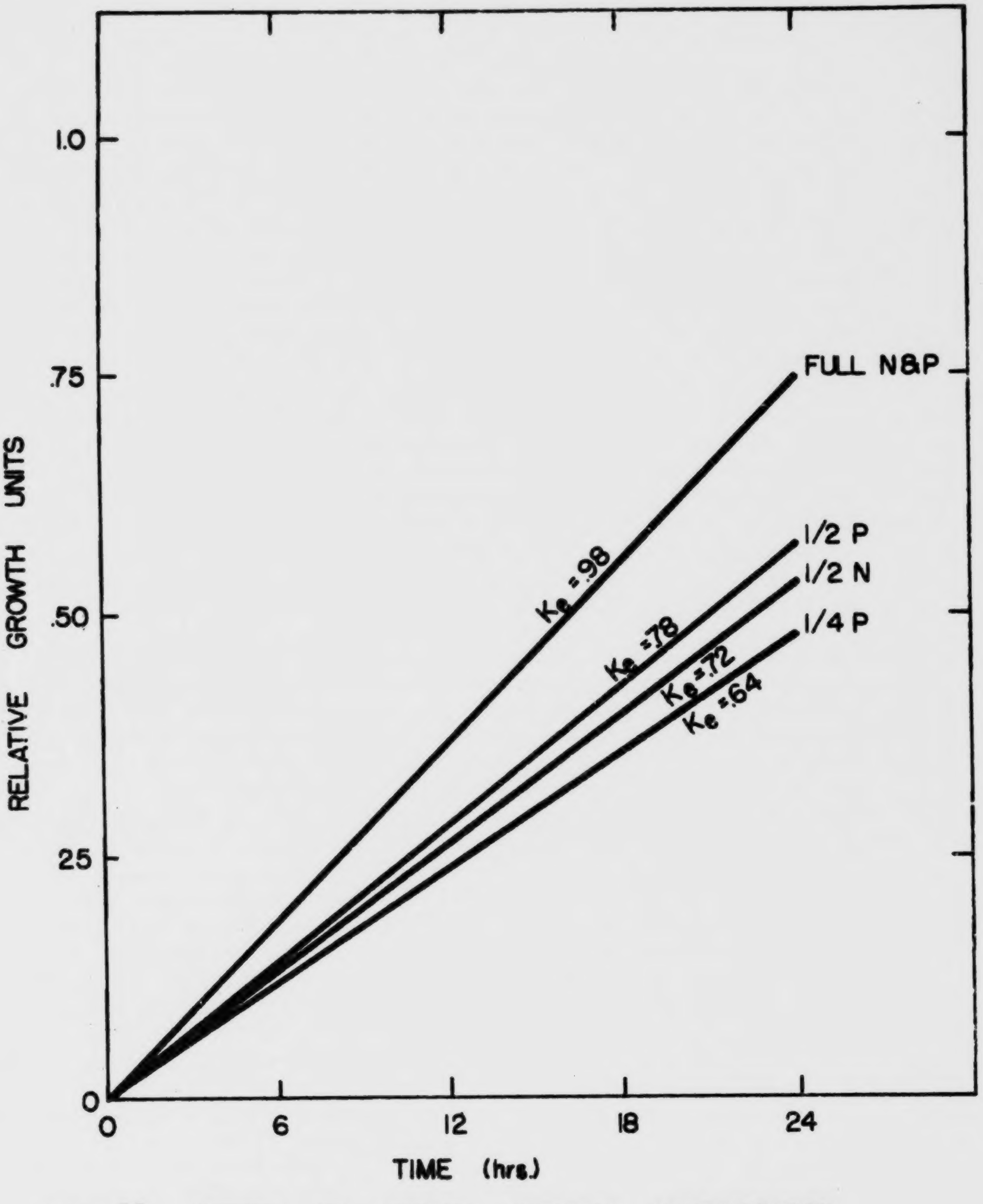


Fig. 23 EFFECT OF VARYING NUTRIENT CONCENTRATION IN ASM (LG) FOR SELENASTRUM CAPRICORNUTUM

substantial reduction in growth rate for the full complement of nitrogen and phosphorus from 1.32 with glass distilled water as diluent to a value of 0.98 with Lake George water as diluent. The substitution of Lake George water as diluent for the Basic ASM medium thus produces a 29% reduction in cell mass production. A similar effect was noted in the growth rate of *Selenastrum capricornutum* in modified tenth Gorham's medium due to the Lake George water diluent. It appears that Lake George water contains some factor inhibitory to the growth of *Selenastrum capricornutum* with greater inhibition prevalent in the Basic ASM medium. The growth rates produced by fractional reductions in phosphorus are comparable to those obtained with these same reductions with glass distilled water. However, the effect of a reduction in nitrogen concentration to the one-half level was more pronounced with Lake George water as diluent than with glass distilled water as diluent. The K rate for the one-half nitrogen level in glass distilled water was 0.96, whereas the K value for Lake George water was 0.72, a 20% decrease in cell concentration due to Lake George water.

The basal medium for experimentation illustrated in Figure 24 was modified tenth Gorham's medium with glass distilled water as diluent. It can be seen that the growth rate for the full complement of nutrients is 1.15 which is considerably higher than the value obtained for Basic ASM with Lake George water and modified tenth Gorham's with Lake George water. The difference noted in K rate for a reduction of nitrogen to the one-half level, that is, a value of 1.03 and a concomitant 25% reduction in cell concentration, was not as pronounced as the reductions noted for a similar decrease in nitrogen level with Basic ASM, using either glass distilled water or Lake George water. When the nitrogen was fractionally reduced to one-fifth its original level there was drastic reduction in the growth rate to a value of 0.40 and a 50% reduction in cell concentration. This indicated that for unimpeded growth a value at least one-half of the full nitrogen concentration is required. For a reduction in phosphorus concentration to one-half the level originally posited a growth rate of 0.60 was obtained as compared to 1.15 for the full complement of phosphorus. This reduction in growth rate represents a decrease of 40% in cell concentration. As noted previously, a reduction to one-half phosphorus level in Basic ASM also produces a 40% change in cell concentration. A predictable result occurred since both

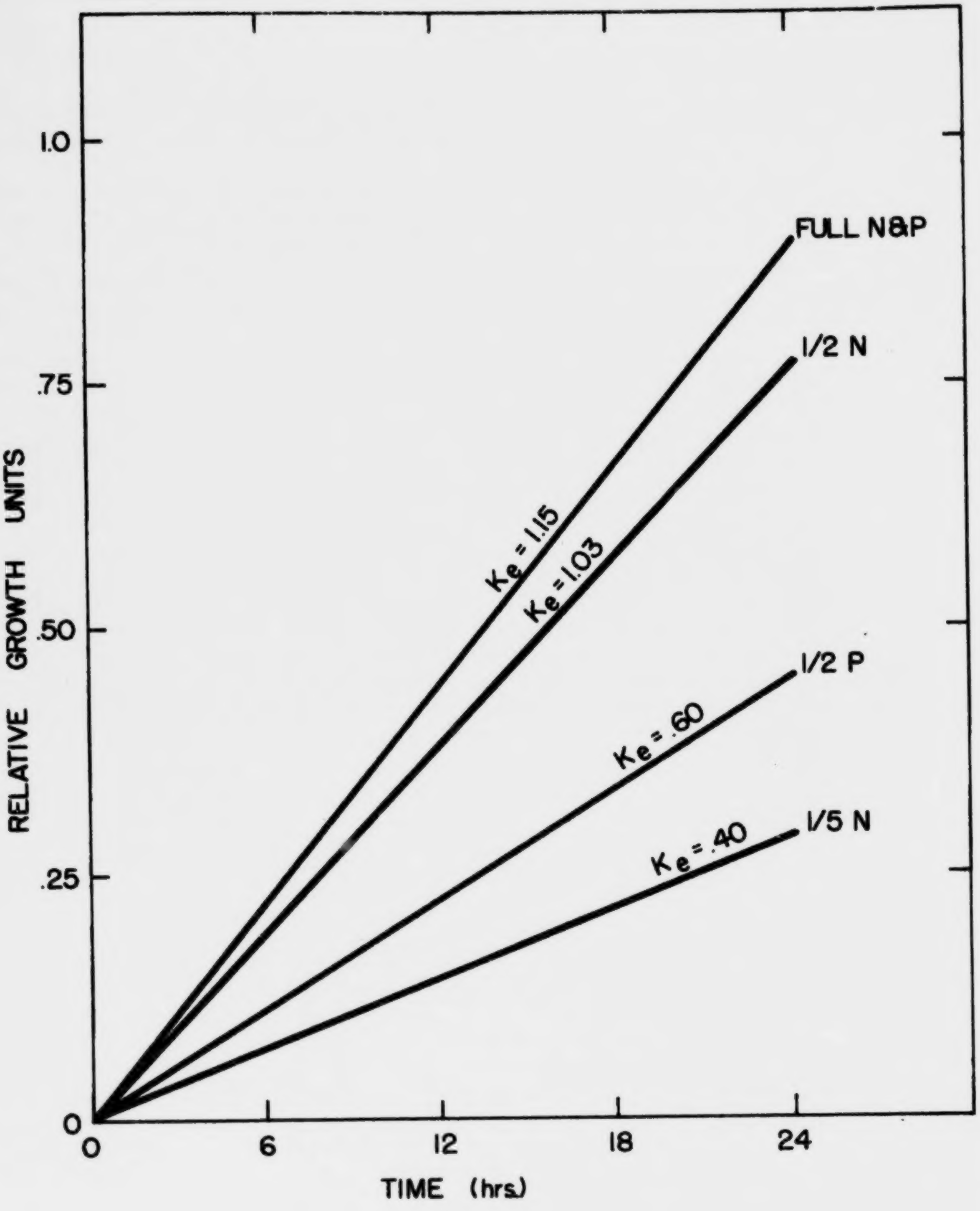


Fig. 24 EFFECT OF VARYING NUTRIENT CONCENTRATION IN MODIFIED .1G (DD) FOR SELENASTRUM CAPRICORNUTUM

media have approximately the same phosphorus concentration and the organism is phosphorus limited in both situations.

Based upon the data presented in Figures 22 and 24 and the relative concentrations of nitrogen and phosphorus in both media, a comparison can be made between the results of Basic ASM with one-half nitrogen and modified tenth Gorham's medium. The Basic ASM medium with one-half nitrogen contains 7 mg/l and the tenth Gorham's medium contains 8 mg/l and approximately the same phosphorus concentration in both media. The K rate obtained for the modified tenth Gorham's medium is 1.15 while the K rate for Basic ASM with one-half nitrogen is 0.96. For a 15% increase in nitrogen level, one obtains 20% greater cell concentration in modified tenth Gorham's medium as opposed to the Basic ASM medium with one-half nitrogen. These proportions again hold when one compares Basic ASM with one-fourth nitrogen and modified tenth Gorham's with one-half nitrogen. The respective growth rates being 0.74 for the former and 1.03 for the latter, again a 20% increase in cell concentration for a 15% increase in nitrogen level. As depicted in Figure 25 higher growth rates were obtained with glass distilled water as diluent than with Lake George water as diluent in both Basic ASM medium and modified tenth Gorham's medium. All curves indicate a predictable effect on the growth rate with increasing concentrations of phosphorus indicated, phosphorus is limiting. However, the limitation is less pronounced with Basic ASM using Lake George water as diluent. It appears that the alga cannot utilize the increased phosphorus concentration because of some inhibitory factor present in Lake George water.

The data presented in Figure 26 are a summation of experimentations with various nitrogen concentrations in modified tenth Gorham's medium with glass distilled water or Lake George water. The curve for modified tenth Gorham's medium was glass distilled water indicates that nitrogen is present in excess in the full medium. If interest were placed on the minimization of nutrient storage and carry over, the nitrogen level could be reduced in this medium to one-half the level presently prescribed. For Basic ASM medium once again the growth rates were higher with glass distilled water than with Lake George water. Increasing concentrations of nitrogen elicited higher growth rates and within the range of concentrations indicated nitrogen was limiting for Basic ASM medium.

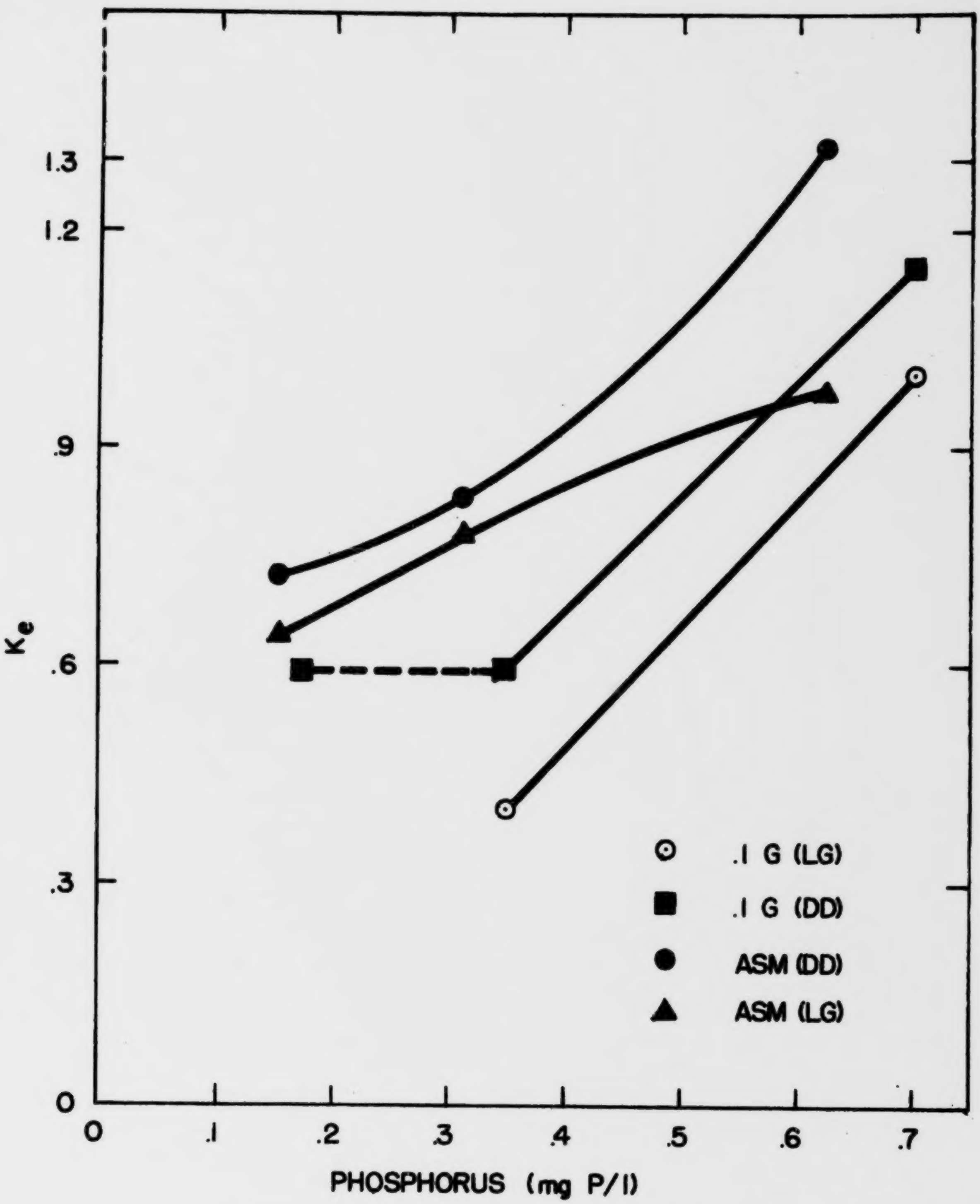


Fig. 25 EFFECT OF VARIOUS PHOSPHORUS CONCENTRATIONS ON VALUES OF K_e FOR SELENASTRUM CAPRICORNUTUM

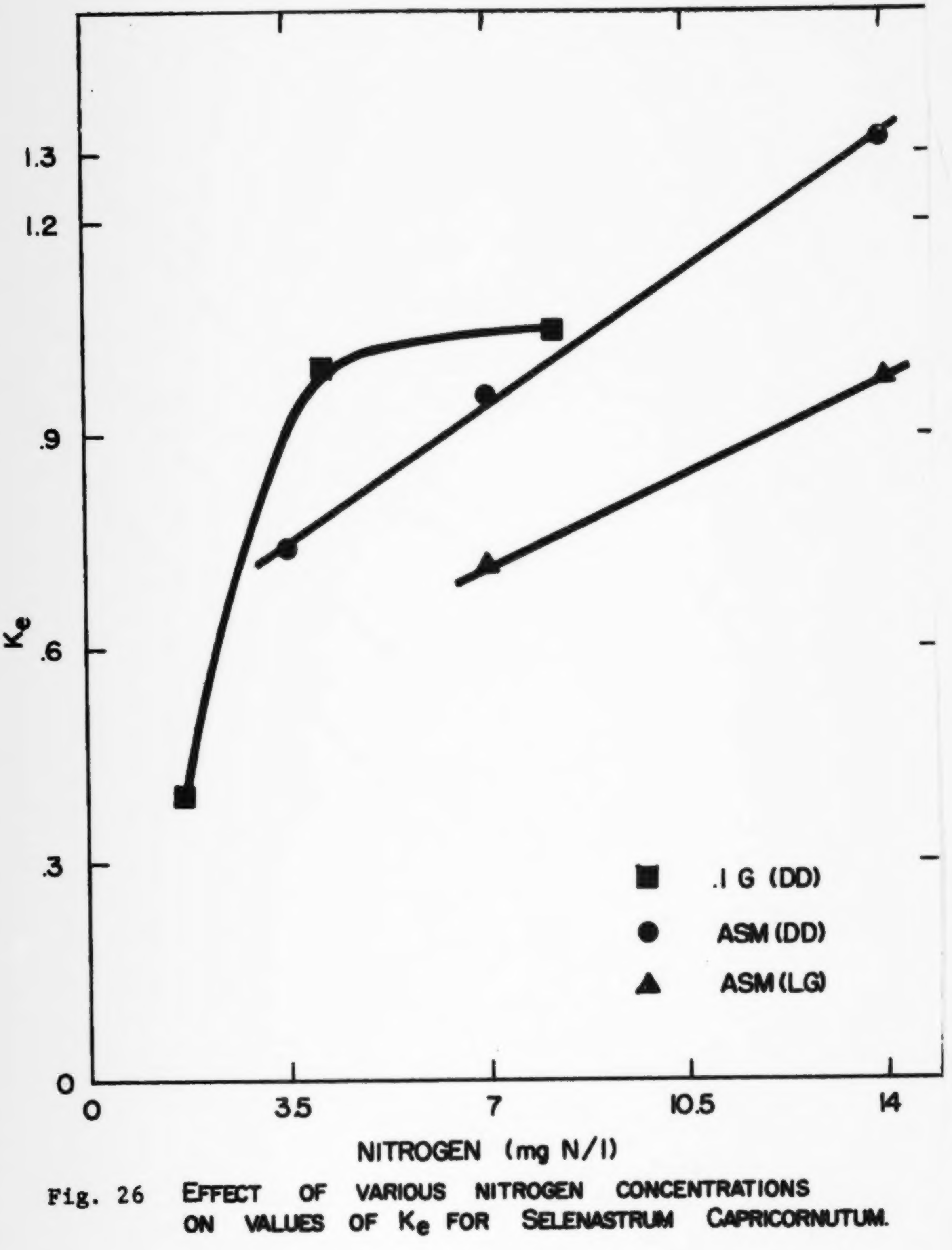


Fig. 26 EFFECT OF VARIOUS NITROGEN CONCENTRATIONS ON VALUES OF K_e FOR SELENASTRUM CAPRICORNUTUM.

In summary it can be stated that there appears to be an inhibition to the growth of the organism as indicated by reduced growth rates when Lake George water is used as a diluent for the basic components of either modified tenth Gorham's medium or Basic ASM medium. To this point, further particular investigation is now being carried on to ascertain the extent and the causative factor of such inhibition. It was also demonstrated that the concentration of nitrogen in modified tenth Gorham's medium may be reduced to one-half the posited level without any significant change in the growth rate of *Selenastrum capricornutum*.

Note: Phosphorous was always tested in the form of phosphate in this latter work.

SECTION VI

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APPENDIX A

COMPARISON OF THIN FILM EVAPORATION AND FREEZE-DRYING METHODS OF CONCENTRATION OF ORGANICS IN WATER

SECTION I: CONCLUSIONS

1. Organic carbon recovery in freeze-drying is less than that in thin film evaporation for all the samples.

2. Relative recovery in freeze-drying is better for sewage samples than for water samples.

3. Less recovery in freeze-drying may be due to (a) absence of flask filters, (b) suction of dry solute into the condenser, (c) use of a large number of flasks, and (d) high vacuum.

4. The chromatograms obtained for thin film and freeze-dried concentrates of the same sample contain an identical number of peaks at about the same elution volume for each peak. This indicates the presence of the same compounds in the two concentrates.

5. The cumulative percentage vs molecular weight curves for the two chromatograms which show the percentage of compounds greater than a certain molecular weight plot out very close to each other for all the samples. The maximum difference between the two curves is less than 2 to 3% at any section. This small difference in the elution profile is believed to be due to instrumental and other errors involved.

6. Any significant polymerization or hydrolysis in either of the concentration techniques employed in this work has not been observed.

7. Although recovery in freeze-drying is less than that in thin film evaporation, the similarity of the chromatograms suggest that there has been no selective removal of a compound (or compounds) from the sample during freeze-drying.

8. Thin film evaporation requires about three times as much time as freeze-drying for concentrating the same amount of a sample.

9. By providing a vacuum pump and a condenser instead of a water aspirator, in thin film evaporation, the rate of evaporation is increased by more than 200 percent.

10. Both methods have merits and demerits but thin film evaporation would be better for concentrating natural waters because of higher organic recovery and freeze-drying would be better for concentrating sewage samples because there is less chance of degradation at lower temperatures.

SECTION II: RECOMMENDATIONS

Based on analysis of the results and experience gained, the following recommendations are made:

1. The precipitate which remains undissolved in 100% HCl, should be treated with dilute NaOH. This might dissolve some of the organic compounds if any are present in the precipitate.

2. The remaining precipitate should be analyzed on a Leco furnace for organic carbon.

3. Future research should also examine the possibility of carrying out thin film evaporation at reduced temperatures and pressures.

4. A larger freeze-drying flask (say 2 liters) would reduce the possible loss of organic carbon due to the use of a large number of flasks.

5. To be absolutely certain about the hydrolysis and polymerization effects of the two methods, the acid filtrate should also be fractionated on the gel column after neutralizing with NaOH and reconcentrating it.

6. A smaller fraction volume, in gel chromatography ie 1.5 ml instead of 5 ml used in this work, may give better defined elution profiles.

SECTION III: INTRODUCTION, HISTORY AND TECHNICAL REVIEW

In recent years considerable research has been done in developing techniques for the determination of organic compounds present in very low concentrations in surface waters. These trace organics are shown to be of significance as causes of taste and odor, color and epidemiological effects (1). The basic methodology for the determination of organic compounds consists of concentrating the water sample followed by analysis of constituents. The main problem, however, is to increase the concentration of organic compounds present, to levels detectable by the instrumental means, without destroying them or altering their chemical composition.

Various concentration methods have been employed to this end, which include carbon adsorption followed by elution with an organic solvent, freeze concentration, solvent extraction, rotary thin film evaporation and freeze-drying. While each of these methods has some advantages and disadvantages, the last two have been studied in some detail in this investigation. Both methods provide a means of concentration by direct removal of water from the sample and do not involve the use of any adsorption material or the addition of an organic solvent.

1. Trace Organics

The trace organics present in natural waters originate from a variety of sources. According to Lysyj et al (2) human activity is the prime source of organic contaminants introduced into waterways, lakes, reservoirs and other water resources. These contaminants may consist of both biodegradable and refractory materials. Sproul (3) in his work on the origin of organics has indicated four general areas from which they originate. These are (1) substances resulting from microbial action, (2) industrial wastes, (3) domestic wastes, and (4) natural runoff. Specific compounds identified from surface waters, as listed by Spicher (4) include:

Pesticides, such as DDT, DDD, methoxychlor, endrin, dieldrin, aldrin, 2,4-D and lindane.

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Detergents, primarily alkylbenzene sulfonates.

Hydrocarbons, including benzopyrene, chrysene, anthracene, phenanthrene, fluoranthene, 10,11 benzofluoranthene, gasoline, kerosene and other fuels.

Other aliphatics and aromatic compounds, including diphenyl ether, pyridine, lutidine, naphthalene, petralin, chloro-ethyl ether, acetophenone, toluene, xylene, ortho-nitrochlorobenzene, ortho-dichlorobenzene, proteins, polysaccharides, tannins and lignins, phenolic compounds and their chlorine derivatives, substituted nitrobenzenes, picolines, fatty acids, nitriles, aromatic ethers, aldehydes, ketones, esters and organic acids.

2. Effects of Trace Organics

Taste and odor problems associated with trace organics are well known. Further, these may interfere with coagulation, foul ion exchange resins (5) and increase activated carbon demand (6) thereby adding to the overall cost of water purification.

Organic contaminants may also add or change the color of the stream, exert excessive BOD, taint fish flesh and upset the microbial purification cycle (7). In industry, where high quality water is required, these trace organics contaminate the product and/or may result in increased production cost and periodic plant shut downs.

3. Detection, Concentration Method

Analytical tests for the detection of organic matter in water and wastewater samples are outlined in the twelfth edition of Standard Methods. These include tests of volatile matter, chemical oxygen demand, biochemical oxygen demand, grease and oil, phenol, alkylbenzene sulfonate and threshold odor. Infrared carbon analysis and gas chromatography are often used to measure the concentration of organic compounds and to identify them. Both of these instruments are rapid and sensitive and yield reliable results for organic concentrations in excess of one mg/l. For lower concentrations of trace organics, as is the case with surface waters, the sample must be concentrated prior to instrumental analysis.

Concentration methods commonly employed are carbon adsorption, solvent extraction, freeze concentration, distillation, ultrafiltration, air drying and reverse osmosis. The other concentration techniques as described by Kammerer (8) are dialysis, coprecipitation and ion exchange. All of the above methods of concentration are still being evaluated.

The carbon adsorption filter evolved by Braus et al (9) is the most widely used method for the concentration of trace organics, but this technique has been shown to have serious disadvantages. Certain compounds may be irreversibly adsorbed or altered on the carbon. According to Hoak (10) the adsorbed organics tend to oxidize on the carbon surface, leading to identification of compounds not originally present. The recovery is also pH dependent, having reduced efficiencies at pH 8.5 or higher (11). Baker (12) has shown that in the carbon filter, selective adsorption and desorption may occur and the organic constituents may undergo chemical and biological modifications.

In solvent extraction the organic compounds are extracted by treating the sample with a solvent, which preferentially dissolves the organic compounds in the sample. This method eliminates the adsorption-desorption problems but it does require the addition of organic solvents and frequently the addition of chemicals like NaCl to reduce the solubility of organic compounds sought.

Ultrafiltration is still in its development stage. The rate of concentration is very slow and equipment is quite costly. Molecules smaller than the membrane pore size pass through it and are lost. The tightest membrane presently available can retain compounds of molecular weight 500 or higher. The concentrates obtained by this method usually require further concentration by other means.

Distillation is not usually employed for concentration of trace organics as it can result in the loss of volatile organic matter and destruction of heat labile compounds.

In freeze concentration the effectiveness decreases with increased dissolved inorganic content. The efficiency depends on a number of factors including rate of cooling, stirring and composition of the original sample (1).

There are two other concentration methods in addition to the ones discussed above. These are rotary thin film evaporation and freeze-drying. Both methods are simple and are used in laboratories in a variety of applications. Their effectiveness in concentrating trace organics in natural or wastewater has never been evaluated. Concentration is effected by the direct removal of water from the sample. The two methods differ mainly in the temperature at which the water vapor is removed. Freeze-drying is generally considered better than thin film evaporation primarily because it is carried at lower temperatures, offering little chance of any degradation of the organic compounds.

4. Objective, Scope and Significance

An evaluation of the literature available on the concentration of trace organics in natural and wastewater indicated that (a) the present knowledge on the effectiveness of thin film evaporation or freeze-drying for concentration is limited and inadequate and (b) research is needed to provide basic information as to the relative effect of the two methods regarding hydrolysis or polymerization of organic compounds. It was the purpose of the study to determine which of the two methods is better when used for the specific purpose of concentrating natural and wastewaters. Both methods of concentration have been studied as to their relative polymerization or hydrolysis effects and their recovery efficiency so far as organic carbon is concerned.

Water samples were concentrated from four different sources. The sources included a high-quality natural water from an oligotrophic lake (Lake George, New York) to the extreme of raw domestic sewage. The water samples were first filtered through membrane filters of 0.45 u pore size. Concentration was carried out by both methods simultaneously. The concentrates were then put on Sephadex G-15 columns for fractionation into different molecular weights (sizes). These fractions were analyzed for organic carbon content on a total carbon analyzer. The two chromatograms thus obtained for each sample have been compared, on a relative basis, for any possible polymerization or hydrolysis reaction.

The control of pollution hinges on our capacity to accurately identify the pollutants. Obviously it is impossible to treat a compound effectively unless its nature is known. Thus, any pollution abatement program has to take into consideration the nature of the compounds present. The obvious approach to the problem of water quality is to identify specific impurities, to develop methods of accurately measuring their presence and concentration in water, and to develop ways and means of removing or lessening their adverse effects. Since some organics may be present in very minute quantity, often below the lower limit of detectability of presently available analytical techniques, these organics have to be concentrated. The method of concentration has to be such that it does not eliminate, alter, or damage the compounds or their distribution in the water sample. This evaluation of the concentration techniques selected will help, to some extent, in the accurate identification of trace organics.

5. Thin Film Evaporation, Freeze-drying and Gel Permeation Chromatography

Thin film evaporation. The history and theory of these processes have been described previously. Discussions of these techniques and review of their concepts can be found within the main body of this text, pp 31-38.

Freeze-drying. Freeze-drying is normally a vacuum drying process in which the water is sublimated from the frozen state. Carman (18) defines it as a process of distillation since a vapor is formed and condensed. Essentially it consists of freezing the sample to be dried or concentrated, keeping it under sufficient vacuum and withdrawing the water vapor which is formed due to evaporation of frozen sample under vacuum.

The process of freeze-drying was first described by Shackell (19) in 1909 for drying biological products. In his process the material was first thoroughly frozen in a salt-ice mixture. The frozen material was then dried *in vacuo* in a desiccator with sulfuric acid as desiccant. In 1921 Swift (20) described a similar technique for drying bacteria from the frozen state, which became standard for some years in many laboratories. However, there was no major improvement in the process till 1931 when Reichel, et al (21) developed a practical procedure for the rapid freezing and rapid dehydration under vacuum of serum in bulk. The serum is shell frozen at -78^{o}C and is connected to a condenser contained in a dry ice-acetone bath (-78^{o}C). The system is also connected to a vacuum source. Rapid sublimation of water in vacuo from its surface keeps the serum in the frozen state. Further improvements in this method have only been concerned with engineering details The use of freeze-drying for the concentration of water and wastewater for analytical purposes has not been extensive. McDonald (22) used it to concentrate sewage and has reported a recovery of 83-100%. McDonald, et al (23) found that chromatograms from the gel filtration of unconcentrated raw sewage and raw sewage concentrated by freeze-drying looked identical, showing no alteration of the compounds when concentrated by this method.

It is interesting to note that the technique of freeze-drying owes its invention and subsequent development to the science of biology. Its use of drying foods was first suggested by Flosdorf and Mudd (24) in 1935. Food technologists are presently working on the development of accelerated freeze-drying methods by the use of microwaves, radiant heat energy, fluidized beds, etc. All these methods aim at heating the frozen product without thawing. However, the rate of dehydration is not a major consideration in concentrating liquids for analytical purposes, where the main aim is not to damage or alter the compounds even slightly.

Gel permeation chromatography. The birth of column chromatography is due mainly to the Russian biologist Tswett (25) who in 1906 separated plant pigment with a calcium carbonate column. The technique passed almost unnoticed until 1931. Adsorption chromatography became standard procedure during the late thirties. Partition chromatography was introduced by Martin and Synge (26) in 1941 which made the separation of hydrophilic compounds like amino acids, polysacchrides and proteins possible for the first time. This technique involves partition of solutes between a liquid and a gas phase. One of these phases is held immobile by an adsorbent material as silica gel in the column.

During the first decade of its development, only liquid chromatography was employed. Following the introduction of gas chromatography by Martin and James (27) in 1952, which affords greater selectivity and resolution, liquid chromatography received a major setback. Gas chromatography, on the other hand, had its own limitations and drawbacks, which included difficulties of measurement of polar substances, macromolecules and preparative isolation of separated fractions. Extensive research conducted in this field has diminished these limitations to a great extent. The method, however, is comparatively very complicated and expensive.

Interest in liquid chromatography was revived with the development of gel filtration, which offered a simple separation method and allowed both the treatment of macromolecules. Separated fractions can readily be used for preparative work which is a major advantage of this technique.

Gel chromatography has a relatively short history. The technique was in its infancy only ten years ago. The introduction of Sephadex Gel by Porath and Flodin (28) has contributed much to its rapid development. Sephadex is a modified dextran, obtained as a reaction product of soluble alkaline dextran and epichlorohydrin. The polymer chains are cross linked by glycerine ether bonds. Other gels available, besides Sephadex, include acrylic gel, polystyrene gel, aerogel, agarose and biogel. Proper pore size, absence of ionizing groups, and, in a given solvent, low affinity for the substances to be separated are the main considerations in the selection of a gel for a particular separation problem. To establish diffusion equilibrium quickly, the gel particles should be as small as possible. Low flow resistance and chemical stability are other desirable properties (29).

Gel forming particles are allowed to swell in the solvent. The amount of swelling depends on the structure of the gel substance and that of the imbibing solvent (30). As a mixture of molecules of various sizes is applied to a gel column, the molecules larger than the largest pores of the swollen gel pass through the mobile liquid phase outside the gel particles because they cannot penetrate into the pores. Smaller molecules are retarded due to the temporary diffusion into the stationary gel phase. Molecules penetrate the network to an extent which, in most cases, is determined by their molecular dimensions and the degree of cross linkage of the gel. The smallest molecules which penetrate the deepest are the last to be eluted. In the gel column, therefore, the larger molecules move faster than the smaller ones and the components of the mixture are eluted in the order of decreasing molecular sizes. According to Porath and Flodin (28) the gel is automatically regenerated after each complete operation.

The use of gel filtration in the field of fractionating organic compounds in natural and wastewater has not been much. Gjessing and Lee (33) employed this technique to separate several samples of river water and concluded that Sephadex columns can fractionate natural water organic matter into a number of distinct fractions. Gel filtration was also employed by Lundblad and Breggard (38) for the separation of low molecular weight components in urine. Zuckerman (39) separated components of several treated and untreated sewage samples on Sephadex columns.

Relatively short time required for separation, the completeness of the separation, good reproducibility, and simplicity of the analytical procedure are some of the major advantages in fractionating the organic matter in natural and wastewater by gel filtration.

Theory of freeze-drying. Carman (18) has discussed the theory of freeze-drying in his paper entitled "Some Basic Principles of Freeze Drying and Molecular Distillation." According to this paper, freeze-drying is closely related to molecular distillation. The conditions generally required for molecular distillation are: a) a pressure above the liquid so low that the vapor molecules which escape from the liquid do not collide with each other or with air and are not reflected back, and b) the boiling, which occurs when the pressure above the liquid is less than the saturation vapor pressure, must be suppressed (eg by freezing the liquid and keeping it frozen during the freeze-drying process). In such cases the vapor will only be formed by the escape of molecules from the surface of the condensed phase.

From the vapor pressure, π, at equilibrium, Knudsen has shown that the rate of collision, R_c, is given by:

$$R_c = \pi\sqrt{\frac{M}{2\pi RT}} \quad \text{in gms/cm}^2\text{/sec}$$

where

M = Molecular weight
R = Gas constant
T = Temperature

If there is no reflection this will be equal to W_o which is the absolute rate of evaporation

$$W_o = \pi \sqrt{\frac{M}{2 \pi RT}} \qquad (1)$$

In the case of reflection instead of reentry

$$W_o = \pi \sqrt{\frac{M}{2 \pi RT}} \qquad (2)$$

If the pressure of the vapor at the interface is P_i instead of π, then the rate of reentry $= \alpha_i \ P_i \sqrt{\frac{M}{2 \pi RT}}$

rate of evaporation W = absolute rate of evaporation W_o - rate of reentry

$$W = W_o - \alpha_i \ P_i \ \frac{M}{2 \pi RT}$$

$$\text{Assuming } \alpha_i = \alpha \text{ then } W = W_o \left(1 - \frac{P_i}{\pi}\right) \qquad (3)$$

When air is present and the vapor escapes to the condensing surface by diffusion through the air layer, then

$$W = \frac{M\bar{D}P}{R\bar{T}X} \ln \left(\frac{P - \pi_c}{P - P_i}\right) \qquad (4)$$

where

P = Total pressure of air in the system

D = Interdiffusional coefficient for vapor and air

π_c = Vapor pressure at the condensing surface

X = Thickness of air layer

$\bar{D}$ and $\bar{T}$ are average values

From equations (3) and (4)

$$W = W_o \left(1 - \frac{P_i}{\pi}\right) = \frac{M\bar{D}P}{R\bar{T}X} \ln \left(\frac{P - \pi_c}{P - P_i}\right) \qquad (5)$$

assuming the condensing surface is so cold that $\pi c = 0$ and if $P \ll \pi$, $P_i \simeq P$

$$\frac{W}{W_o} \simeq (1-P/\pi) \qquad (6)$$

if $P \gg \pi$, $P_i \simeq \pi$ So

$$\frac{W}{W_o} \quad \frac{M\bar{D}P}{W_o \; RTX} \ln \left(\frac{P}{P-\pi}\right) \qquad (7)$$

Depending on the conditions prevailing in the system, equation (6) or (7) may be used to find the rate of evaporation in the freeze-drying process. Usually the pressure in the system is maintained at only a few microns and very few air molecules are present as compared to vapor molecules. In this case equation (6) is applied. It is seen that rate of evaporation will be increased if the total pressure of air in the system is decreased.

Theory of gel filtration. It has been shown that in gel filtration, the molecules emerge from the column in the order of decreasing molecular weights (sizes). This simple fact has prompted many researchers to derive a relationship between the molecular weight of a molecule and its elution volume. Granath and Flodin (34) found alinear relationship between the logarithm of the molecular weight and the ratio of the elution volume to the total bed volume for a number of dextrans. Whitaker (35) in his experiment on Sephadex G-100, observed a linear relationship between the logarithm of the molecular weight of a protein and the ratio of its elution volume, V_e, to the void volume, V_o, of the column. He also found that, within limits, the ratio V_e/V_o was independent of the solute concentration, sample size and dimensions of the column, but it was found to be temperature dependent for several proteins.

A quantitative expression to find the molecular weight from elution volumes has been derived by Porath (36). Considering the cavities in the swollen gel available for penetration of the protein molecule of radius r, as conical with radius R, he found that

$$k_d = k(1- \frac{r}{R})^3 \qquad (9)$$

where k_d is the distribution coefficient

As R^3 is closely related to solvent regain S_r

$$R^3 \sim (S_r - \alpha)$$

and r is proportional to the square root of molecular weight M equation (9) may be written as

$$k_d = k\ (1 - k_1 \frac{M^{1/2}}{(S_r - \alpha)\ 1/3})^3 \qquad (10)$$

He also found a value of 1.64 for K for the Sephadex gels and 0.8 for α · K_1 was found to be close to 0.012. K_d can be found from the elution volume such as

$$k_d = \frac{V_e - V_o}{V_i} \quad \text{if there is no adsorption.}$$

V_i is the inner volume and is approximately equal to solvent regain.

Since all quantities except M in equation (5) are known or can be found experimentally, M can easily be calculated. Working along similar lines, Squire (37) has derived a different equation. His treatment is based on the assumption that the cavities available to the protein molecule consist of a mixture of cones, cylinders and crevices. According to him

$$\frac{V_e}{V_o} = (1 + g\ (1 - \frac{M^{1/3}}{C^{1/3}}))^3 \qquad (11)$$

where C corresponds to the molecular weight of the smallest protein that cannot enter the gel and g is a constant representing the geometric relationship between the three types of cavities in a given gel. Equation (11) can also be written as

$$M^{1/3} = \frac{C^{1/3}}{g}\ (1 + g - (\frac{V_e}{V_o})^{1/3}) \qquad (12)$$

Values of $C^{1/3}$ and g for a particular gel column are determined experimentally from a plot of $(V_e/V_o)^{1/3} - 1$ vs $M^{1/3}$. Once the values of C and g for a column are known, equation (11) can be used to find the elution volume for a particular compound of known molecular weight, or vice versa.

SECTION IV: PROCEDURES AND INVESTIGATIONS

Methodology

1. Sample Collection and Storage

The sample in each case was collected in a 20-liter plastic carboy which had been repeatedly and thoroughly cleaned and washed. It was also rinsed two or three times with the sample source. The carboy was filled with the sample, capped and brought to the laboratory where it was placed in the cold room. The cold room temperature is kept between 3^o - 5^oC.

The following samples have been taken:

Sample Type	Location	Date
Lake George Water	Near the dock at Smith Bay	9/5/70
Saratoga Lake Water	Dock, 1.5 miles north of Public Beach	11/29/69
Clifton Knolls effluent	At Treatment Plant after chlorination	3/26/70
Elnora raw sewage	At Treatment Plant Clifton Knolls	4/16/70

The sample was filtered through a membrane filter of 0.45 u pore size and 142 mm diameter on the same day. The plastic carboy in which the filtered sample was stored in the cold room was also properly cleaned and rinsed.

Concentration of the sample was started simultaneously by both methods. The thin film method took more time than freeze-drying.

2. Procedure for Thin Film Evaporation

The motor is securely fixed to a ring stand such that the teflon shaft is inclined at about 30^o to the horizontal. The evaporating dish is half filled with water and is placed on the hot plate. The round bottom flask is then fixed to

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the tapered end of the shaft. Two liters of the water sample was put in the feed bottle. About 500 ml sample was added to the evaporating flask by means of the feed tube and the system was connected to a water aspirator. When the water started boiling in the flask, the motor was switched on to rotate the flask. The thermostat was set to give the desired temperature in the water bath. Water level in the evaporating dish was maintained with the help of a constant level device.

Sometimes, due to insufficient vacuum, the water in the flask did not boil when the motor was stopped. Usually it was the ball joint that was the trouble spot and needed readjustment and tightening.

A little grinding action at the ball joint sometimes sent very small teflon grinding into the flask, which looked like bacterial growth. When checked under a microscope, its exact nature was revealed. Since the concentrate was passed through a 0.45 u membrane filter before applying it to the gel filtration column, or to the carbon analyzer, the teflon particles did not affect the results in any way.

A continuous feed into the evaporating flask was also attempted by adjusting the pinch clamp on the tygon tubing. The rate of evaporation was so small that proper adjustment of the pinch clamp could not be made. The feed was, therefore, let intermittently into the flask.

3. Freeze-drying

The center well of the freeze-dryer is filled to 1/3 its depth with acetone. Dry ice is added to the acetone in very small pieces one at a time so that the acetone does not overflow the vessel as CO_2 escapes when the acetone is cooled. More dry ice is added when the bubbling stops. All the quickseal valves are closed, oil level in the vacuum pump is checked and the pump is started.

The sample to be concentrated is put in a freeze-drying flask of 1.2 liter capacity. It is then put in a dry ice/acetone bath and rotated continuously at about 80 rpm for "shell" freezing of the sample. The flask is kept in a slanting position while rotating.

After all the liquid in the flask is completely frozen, it is attached to one of the ports on the manifold. The quickseal valve is opened and a high vacuum is reestablished in the system by the pump. It has been observed that when the pressure is more than 100 u of Hg, it is difficult to maintain a sample in the frozen state during the drying operation. Usually the pressure is kept less than 50 u of Hg.

Although the capacity of each freeze-drying flask is 1.2 liters, only about 500 ml of the sample can be concentrated in a flask at a time. Sample volumes of more than 650 ml showed a tendency of melting adjacent to the walls and the bottom of the flask. This tendency was more pronounced at the bottom of the flask. It appears that the ice thickness in the flask becomes too great to conduct all the heat, supplied by the atmosphere, to the evaporating surface. No additional heat is supplied to the system but normally the ambient room temperature combined with the condensation of moisture in the room on the external surface of the flask supplies just the right amount of heat to efficiently sublimate the ice content of the sample.

After all the flasks have been frozen, some of the acetone is taken out of the center well and dry ice is added in its place. For overnight operation an extender is placed on the center well and filled with the dry ice. It is then covered.

At the completion of the operation, the quickseal valves are turned only 1/4 turn clockwise to retain vacuum in the flasks and the vacuum pump is stopped. The vacuum is released by opening a valve which is not attached to any flask. The flasks are then removed along with the quickseal valves and the vacuum in each flask is released very slowly so as not to disturb the powdered concentrate in the bottle. The sample is usually not concentrated to complete dryness. If the apparatus is not to be reused immediately, it is better to release the vacuum in the flask after the ice has melted.

Although the ports were internally baffled, ice clogging was noticed occasionally on a few ports which resulted in the melting of the sample. The concentrated sample was filtered through 0.45 u membrane filter. Acid filtrate is obtained by dissolving the precipitate in 1:10 dilute HCl and filtering it through the membrane filter.

4. Procedure for Gel Chromatography

A calculated amount of Sephadez G-15 was swollen in double-distilled water in the cold room for 24 hours. The column was assembled vertically and a stoppered funnel was fixed onto the upper end. A flow adaptor was fixed at the upper end. A flow adaptor was fixed at the lower end and the outlet was closed. The column was then filled with the solvent. The swollen gel along with some solvent was put into the funnel when a layer of about 15 to 20 cm of the gel had settled, the outlet was opened and the solvent was allowed to flow out slowly. More gel came into the column and was packed gradually. The verticality of the column was checked with a plumb bob. The eluent reservoir bottle was connected to the top of the column and flow of buffer was maintained for 36 hours so that the gel is properly settled.

To apply a sample to the column, the eluent above the gel packing was removed with the help of a 10 ml syringe. Whatever was left was allowed to penetrate the gel by keeping the outlet open. A measured amount of sample was then applied to the column. Flow rates through the column were maintained by adjusting the position of the eluent reservoir. Five ml fractions were collected with the help of an LKB fraction collector, and were analyzed on the carbon analyzer for organic carbon.

A chromatogram is obtained by plotting the organic carbon (mg/l) as ordinate and elution volume or fraction number as abcissa. The elution volume of a particular compound is the effluent volume as measured from the application of the sample to the elution of the component at maximum concentration.

5. Column Standardization

The gel column must be standardized before any sample is applied to it for analysis. The Sephadex G-15 column was standardized using a solution of blue dextran (a dextran with a blue dye chemically bound to it - molecular weight approximately 2 x 10^6), raffinose (molecular weight 594.32), maltose (molecular weight 360.32), glucose (molecular weight 180.16) and sodium chloride (molecular weight 58.44). The homologous compounds were selected as standards because they eliminate the possibility of interference due to different configurations of other molecules. Another reason is that carbohydrates are also present in the samples to be analyzed. The above compounds were dissolved in double-distilled water. and the solution was filtered through 0.45 u membrane filter before applying it to the column.

Phosphate buffer was used as eluent. It consisted of the following compounds:

Potassium dihydrogen phosphate	225.7 mg/l
Dipotassium hydrogen phosphate	577.4 mg/l
Disodium hydrogen phosphate	886.8 mg/l

The pH of the buffer was 7.6.

Length of the gel column was 90.5 cm and its diameter was 2.5 cm. The rate of flow through the column was 50 ml/hr. The effluent was collected in 5 ml fractions which were analyzed on the carbon analyzer. The chromatogram obtained is shown in Figure 1.

The molecular weight of the sample is plotted against its elution volume on a semilog paper and a calibration curve is obtained, as shown in Figure 2. The elution volume has been shown to be a linear function of the logarithm of the molecular weight over a considerable range.

6. Procedure for Organic Carbon Determination

The carbon analyzer was calibrated for the range 0 mg C/l to 100 mg C/l using oxalic acid ($HO_2CCO_2H \cdot H_2O$ molecular weight 126.07) standard solutions. These solutions were prepared in carbon dioxide free, double-distilled water in carbon concentrations of 2, 4, 10, 20, 40, 80 and 100 mg/l. By use of the sensitivity switch the instrument was adjusted to give the maximum reading when 40 ul of 100 mg C/l standard solution was injected. Readings corresponding to each standard concentration were obtained at that gain control and a calibration curve was obtained. The calibration curve was not linear for this instrument.

Each 5 ml fraction was acidified to a pH of 2.0 with 2N HCl and purged of CO_2 with nitrogen gas for five minutes at a gas flow rate of about 500 cc/minute. Fourty ul of the sample was then injected with a Hamilton SK 148, 50 ul syringe for organic carbon analysis. The Beckman furnace

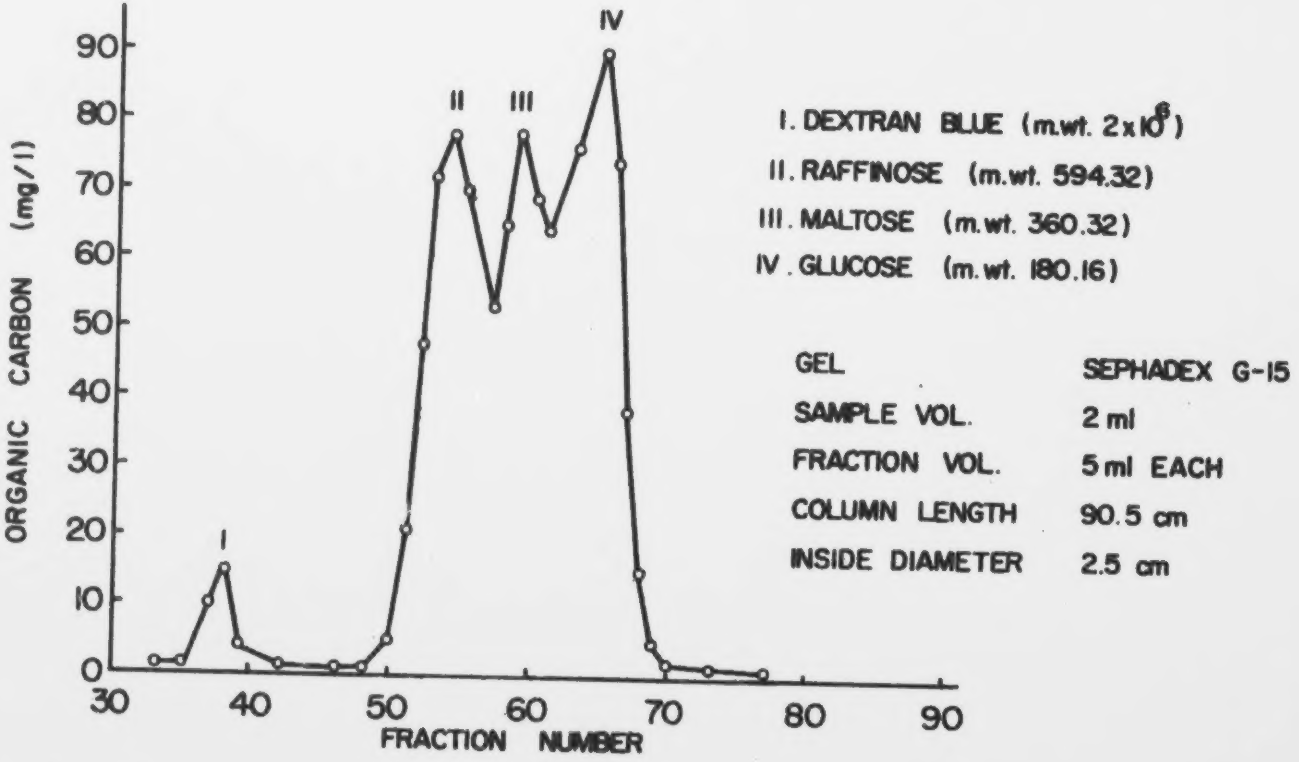

Fig. 1 CHROMATOGRAM OF STANDARD SOLUTION ON SEPHADEX G-15 COLUMN WITH PHOSPHATE BUFFER.

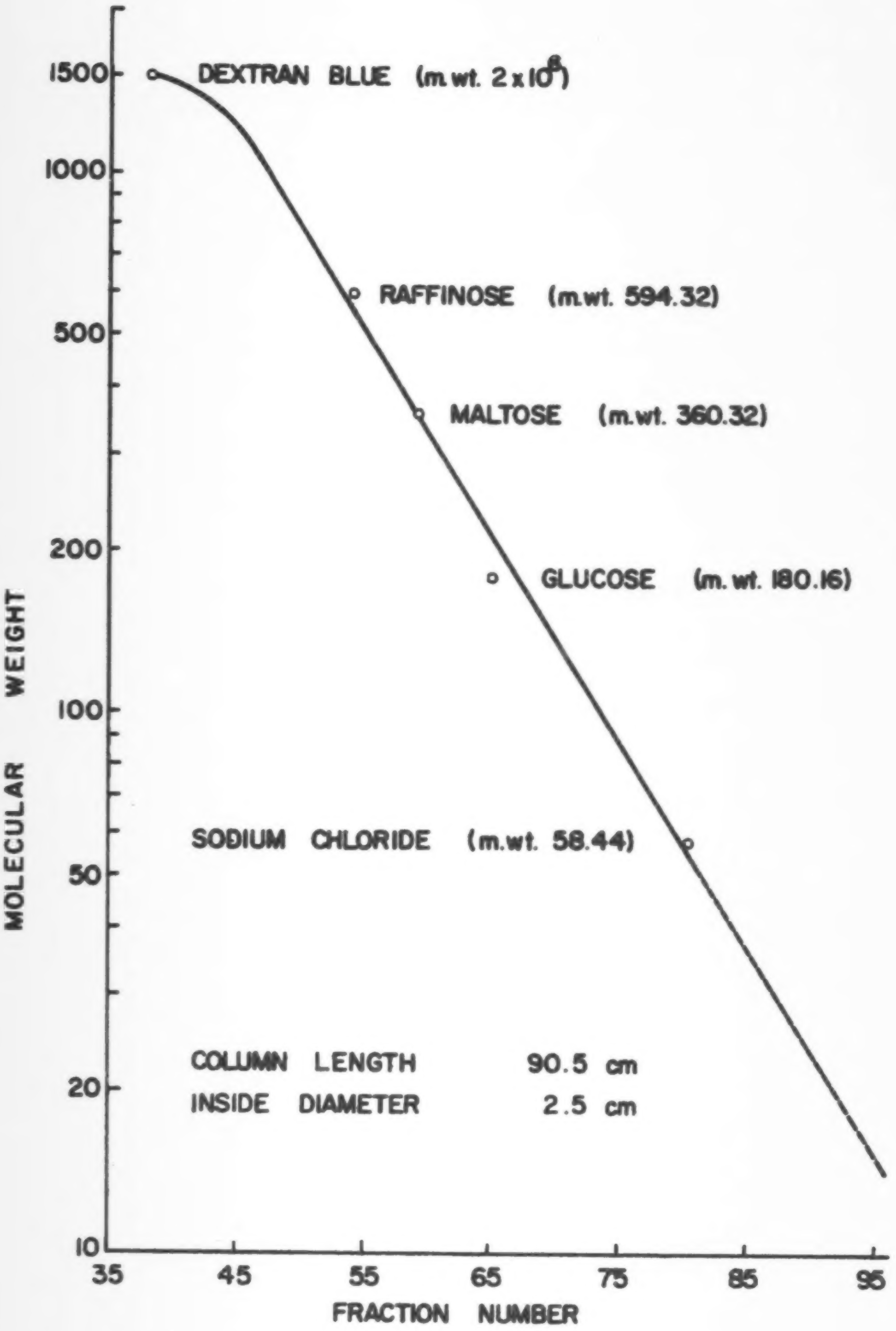

Fig. 2 CALIBRATION CURVE FOR SEPHADEX G-15 COLUMN

had arrangements for measuring total as well as inorganic carbon separately but the inorganic channel did not give consistent readings. The above procedure of purging inorganic carbon was, therefore, employed.

Results and Data Analysis

1. Saratoga Lake Water

Concentration. About 20 liters of Saratoga Lake water were collected on November 24, 1969 from the east shore, about 1-1/2 miles from the south end of the lake. The temperature of the water sample as collected was 12°C and its pH was 8.35. The sample was filtered through a 0.45 u membrane filter. Thin film evaporation was started with 2 liters of sample in the feed bottle. More sample was added when the initial feed had evaporated. A total of 5 liters was concentrated by this method to 141 ml, a concentration factor of 35.5. Although the sample was colorless, the concentration was slightly colored and appeared to be somewhat viscous in the evaporating flask. Therefore, further concentration was not carried out. On filtering, however, the viscous appearance turned out only to be due to a slight color effect. The concentrate was not viscous.

The freeze-drying was started in 8 flasks. Five hundred ml of the sample was put in each flask, shell-frozen in a dry-ice/acetone bath and the flask was connected to the manifold. The freeze-dryer was kept running at night but the V-belt of the vacuum pump broke and the frozen samples melted in the flasks. The freeze-drying was again started after replacing the V-belt in the morning. The concentrated contents of the 8 flasks were transferred into 2 flasks and again freeze-dried. The final concentrate obtained was 62 ml (concentration factor of 64.5) and it was colorless. Table 1 represents the concentration and recovery data for this sample.

The organic carbon recovery in the freeze-drying method is only 60% of that in the thin film evaporation. The recovery has been calculated on the basis of total organic carbon recovered in each method, which includes both the concentrate and the acid filtrate.

Another sample of Saratoga Lake water was obtained and concentrated by both methods. A relative recovery of 79% was

TABLE 1

CONCENTRATION AND ORGANIC RECOVERY DATA

SARATOGA LAKE WATER

SAMPLE NO. 1

Concentration Data	Thin Film Evaporation	Freeze-drying
Initial volume of the sample	5 liters	4 liters
Final volume (concentrate)	141 ml	62 ml
Volume of the acid filtrate	16 ml	15 ml
Concentration factor (based on concentrate volume only)	35.5	64.5
Temperature of the water bath	34.5°C-37°C	-----
Vacuum pressure	40-60 mm of Hg	20-50 u of Hg
Organic Recovery Data		
Organic carbon content		
Concentrate	100 mg/l	100 mg/l
Acid filtrate	30 mg/l	52 mg/l
Total organic content of the sample (as recovered)	14.580 mg	6.980 mg
Total organic carbon recovered per liter of the sample	2.916 mg	1.745 mg
Percentage recovery (based on 100% recovery in thin film evaporation)		
Total including acid filtrate	100%	60%
Only concentrate	100%	55.5%

obtained this time. The concentration data for this sample are shown in Table 2. The concentration by freeze-drying was carried out on a "Freeze-mobile" (The Virtis Company, Inc.) and not on the bench model freeze-dryer as previously described.

Chromatographic studies. The concentrates obtained by both methods were applied to the gel column packed with Sephadex G-15 to fractionate the organic compounds present. Phosphate buffer was used as eluent. The fractions were analyzed on the carbon analyzer. The readings for individual fractions are shown in a later section, (pages 255-262). The chromatograms obtained are shown in Figures 3 and 4, for the rotary thin film and freeze-dried concentrates, respectively. The chromatographic data are shown in Table 3.

The two chromatograms look very much alike, although there are some minor differences. The peaks appear at the same elution volume in both chromatograms and are identical in number. This shows the presence of the same compounds in both the concentrates and, therefore, no relative hydrolysis or polymerization.

This, in fact, is an oversimplification of the statement (analysis). The necessity to compare the two chromatograms in some other way is obvious. It was decided to plot the cumulative percentages of the compounds eluted with successive fractions. As the elution volume is a linear function of the logarithm of the molecular weight, the graph (Figure 5) obtained gives, at a glance, the percentage of compounds in the sample which are greater than a certain molecular weight. In this way the comparison is made of the compounds in the two concentrates as they are eluted from the gel column. For example, the thin film concentrate has 16% of the compounds with apparent molecular weight greater than 1,500, whereas the freeze-dried concentrate also has 16%; similarly for apparent molecular weight greater than 350, the corresponding figures are 85% and 83% for thin film and freeze-dried concentrates, respectively.

The two percentage curves (Figure 5) for the thin film concentrate and the freeze-dried concentrate plot out very close to each other and differ not more than 2.5% at any section. This small difference is hardly of any importance, considering the

TABLE 2

CONCENTRATION AND ORGANIC RECOVERY DATA

SARATOGA LAKE WATER

SAMPLE NO. 2

Concentration Data	Thin Film Evaporation	Freeze-drying
Initial volume of the sample	2.5 liters	2.5 liters
Final volume (concentrate)	16 ml	17 ml
Volume of the acid filtrate	55 ml	28 ml
Concentration factor (based on concentrate volume only)	156	147
Temperature of the water bath	33^oC	-----
Vacuum pressure	40-60 mm of Hg	20-50 u of Hg
Organic Recovery Data		
Organic carbon content		
Concentrate	651 mg/l	390 mg/l
Acid filtrate	24 mg/l	95 mg/l
Total organic content of the sample (as recovered)	11.736 mg	9.290 mg
Total organic carbon recovered per liter of the sample	4.694 mg	3.716 mg
Percentage recovery (based on 100% recovery in thin film evaporation)		
Total including acid filtrate	100%	79.2%
Only concentrate	100%	61.75%

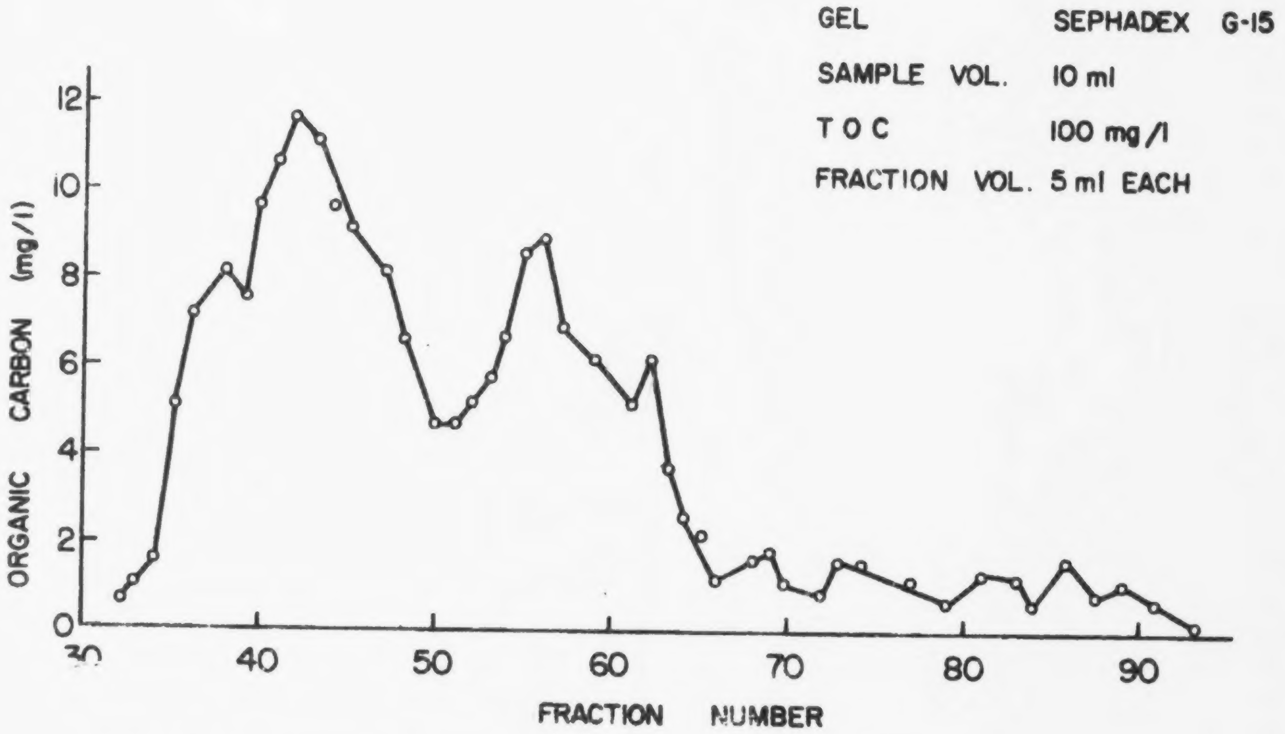

Fig. 3 Chromatogram of Saratoga Lake water thin film concentrate

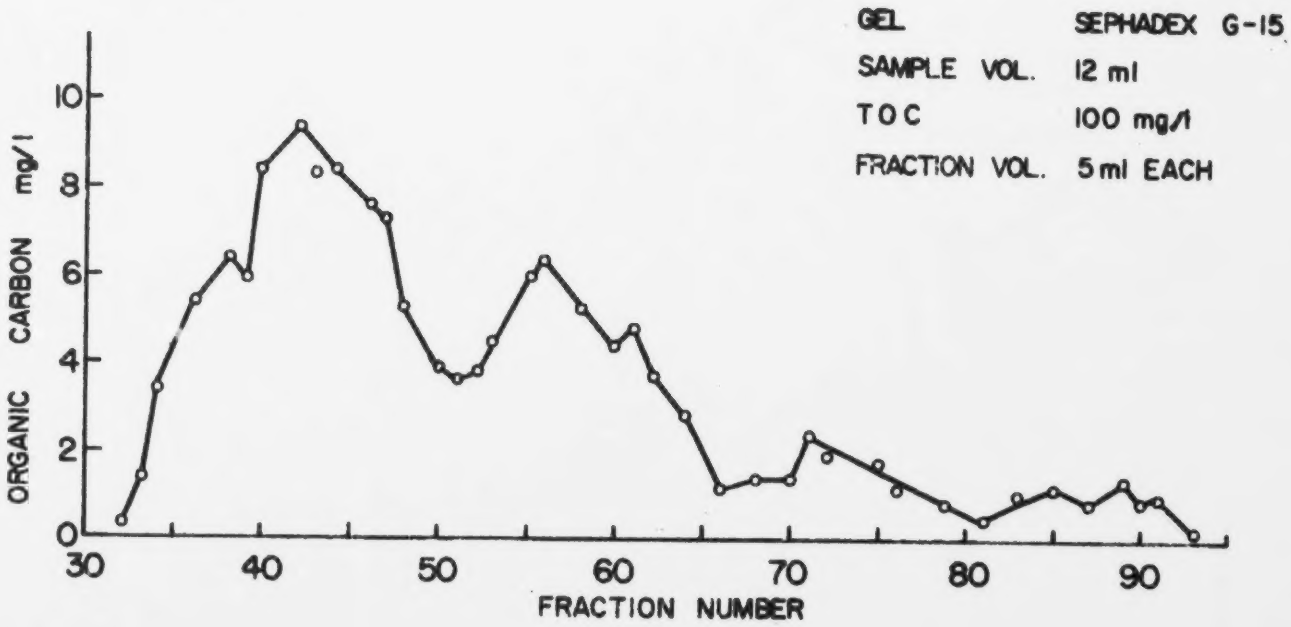

Fig. 4 Chromatogram of Saratoga Lake water freeze dried concentrate

TABLE 3

CHROMATOGRAPHIC DATA FOR SARATOGA LAKE WATER

SAMPLE NO. 1

Preliminary Data	Thin Film Evaporation	Freeze-drying
Volume of the sample applied	12 ml	10 ml
Volume of gel column	444 cm^3	444 cm^3
Sample volume as percentage of column volume	2.70%	2.25%
Organic content of sample	100 mg/l	100 mg/l
Total organic carbon applied to column	1.20 mg	1.0 mg
Rate of flow through the column	44 ml/hr.	28 ml/hr.
Molecular Weight Distribution Data		
Compounds greater than apparent molecular weight 1500 in the sample	16%	16%
Compounds greater than apparent molecular weight 1200 in the sample	43%	43%
Compounds greater than apparent molecular weight 700 in the sample	60%	60%
Compounds greater than apparent molecular weight 350 in the sample	85%	83%
Compounds greater than apparent molecular weight 180 in the sample	93%	91%

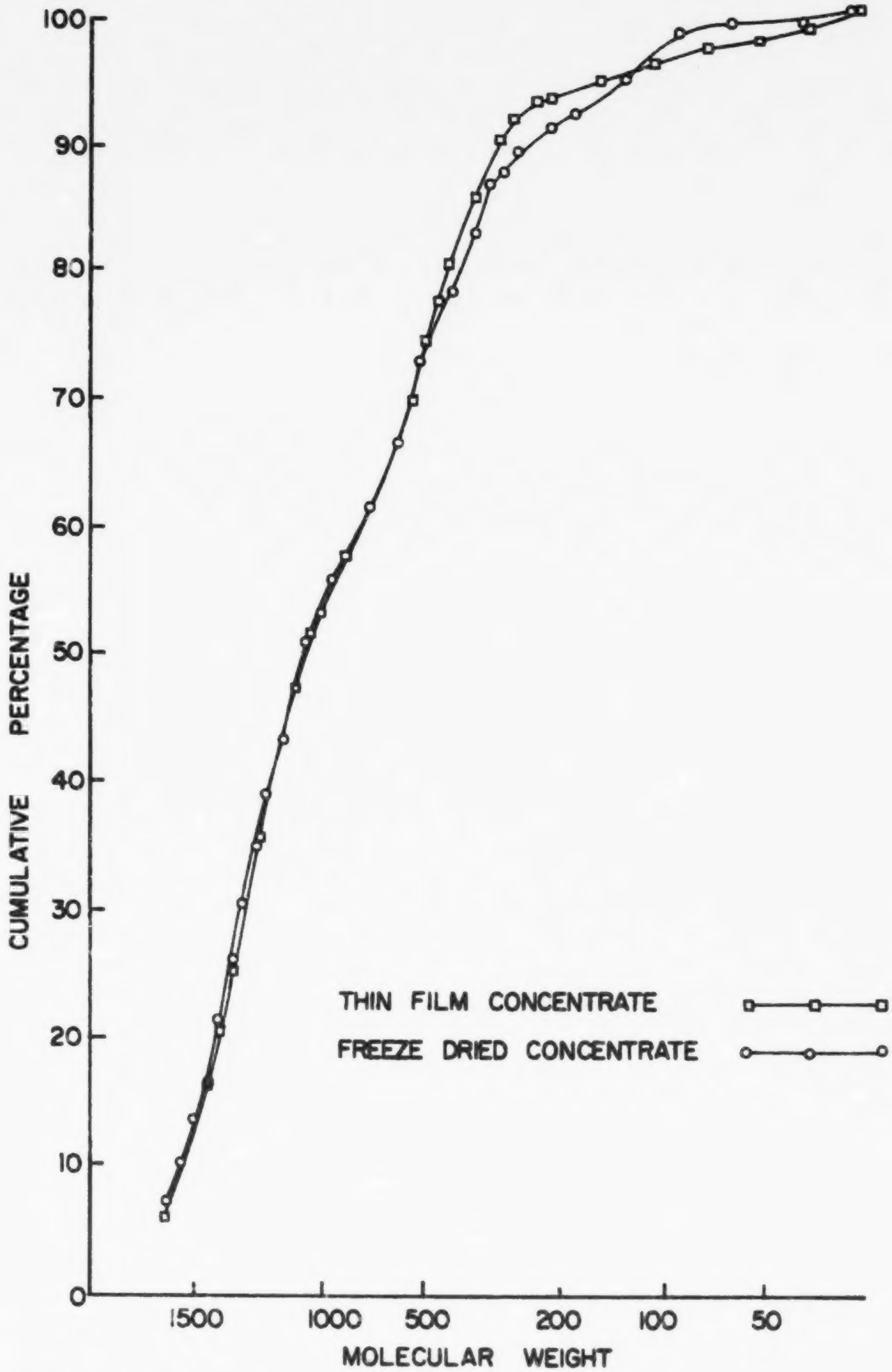

Fig. 5 PERCENTAGE CURVES FOR CHROMATOGRAMS OF SARATOGA LAKE WATER

instrumental and other errors involved. Any significant hydrolysis or polymerization of the compounds while the sample is concentrated by either of the two methods is, therefore, ruled out.

As is seen from Figure 5, the compounds of molecular weight less than 200 constitute only 10% of the sample and 60% of the compounds are with molecular weight greater than 700.

2. Clifton Knolls Sewage Effluent

Concentration. The Clifton Knolls Sewage Treatment Plant is located about fifteen miles northwest of Troy, New York. The sewage is treated by the contact-stabilization process and the effluent is chlorinated before it is discharged into the ponds. The chlorinated effluent sample was collected in a 20 liter plastic carboy on March 26, 1970. Twenty ml of $HgCl_2$ (stock solution with 40 gms $HgCl_2$/liter) was added to it to eliminate the possibility of any biological degradation during storage. The temperature of the effluent sample was 20°C and its pH was 7.2. The sample was filtered twice, first through a coarse filter and then through 0.45 u membrane filter.

The freeze-drying was started with 2.5 liters of the sample in 7 flasks. After one day, the flasks were removed and ice remaining in the flasks was allowed to melt. When 2.5 liters more of the sample were added, the concentration was again started. The concentrate from all the flasks was transferred into one flask and was finally concentrated to 32 ml (a concentration factor of 156.2). Acid filtrate obtained by dissolving the precipitate in 1:10 HCl was 22 ml. As the sample was concentrated too much, a large amount of material had precipitated in the flask.

Five liters of the sample was simultaneously concentrated by thin film evaporation. The problem of heavy deposition along the walls of the evaporating flask (near the top) was encountered while concentrating this sample. Although some deposition is always found while concentrating sewage sample and has also been reported in the literature, in this case the addition of $HgCl_2$ had also contributed much to this deposition. The concentrate obtained was 91 ml (a concentration factor of 54.9) and the acid filtrate was 20 ml.

The organic carbon recovery in the freeze-drying method was 90.8% of that in the thin film evaporation. The concentration and organic recovery data are shown in Table 4. This comparatively higher recovery in freeze-drying is partly due to the poor performance of the thin film evaporator in this particular case. The deposition along the walls was extensive, sticky, and difficult to dissolve completely in dilute (1:10) HCl. It is believed that some organic carbon must have been left in the flask along with the deposition which could not be dissolved even after rotating the flask with dilute HCl for about an hour. If acid filtrates are excluded the relative organic carbon recovery in freeze-drying comes to only 76.8%.

The acid filtrate obtained from freeze-drying contains 29.6% of the total organic carbon recovered. In thin film evaporation, it is only 16.5%. Comparatively greater amounts of organic carbon in acid filtrate from freeze-drying may partly be due to a larger concentration factor in this method, which is 156.2 as compared to 54.9 in thin film evaporation.

Chromatographic studies. A 10 ml aliquot of the freeze-dried concentrate was put on the G-15 Sephadex column. Phosphate buffer was used as eluent. The flow rate through the column was 46 ml/hour. The 5 ml fractions as analyzed on the carbon analyzer are shown in a later section. The chromatogram is shown in Figure 6.

The thin film concentrate did not have sufficient organic carbon in it and thus was further concentrated before applying it to the gel column. The volume of the sample applied to the column was 5 ml. The chromatogram is shown in Figure 7. The individual readings for the 5 ml fractions as analyzed on the carbon analyzer are shown in a later section. The general chromatographic data for the two concentrates are shown in Table 5.

Both the chromatograms have two major peaks at apparent molecular weight - 1,500 and 290; and two small peaks at molecular weight - 600 and 180. Another good-size peak appears at the end of the elution. According to the calibration curve, the molecular weight of the compound should be about 30. Possibly the nature of the compound is such that it has stayed in the gel for more than what is theoretically required according

TABLE 4

CONCENTRATION AND ORGANIC RECOVERY DATA

CLIFTON KNOLLS EFFLUENT

Concentration Data	Thin Film Evaporation	Freeze-drying
Initial volume of the sample	5 liters	5 liters
Final volume (concentrate)	91 ml	32 ml
Volume of the acid filtrate	20 ml	22 ml
Concentration factor (based on concentrate volume only)	54.9	156.2
Temperature of the water bath	32°C-35°C	
Vacuum pressure	40-60 mm of Hg	50-100 u of Hg
Organic Recovery Data		
Organic carbon content		
Concentrate	126 mg/l	275 mg/l
Acid filtrate	114 mg/l	168 mg/l
Total organic carbon in the sample (as recovered)	13.768 mg	12.496 mg
Total organic carbon recovered per liter of the sample	2.754 mg	2.499 mg
Percentage recovery (based on 100% recovery in thin film evaporation)		
Total including acid	100%	90.8%
Concentrate only	100%	76.8%

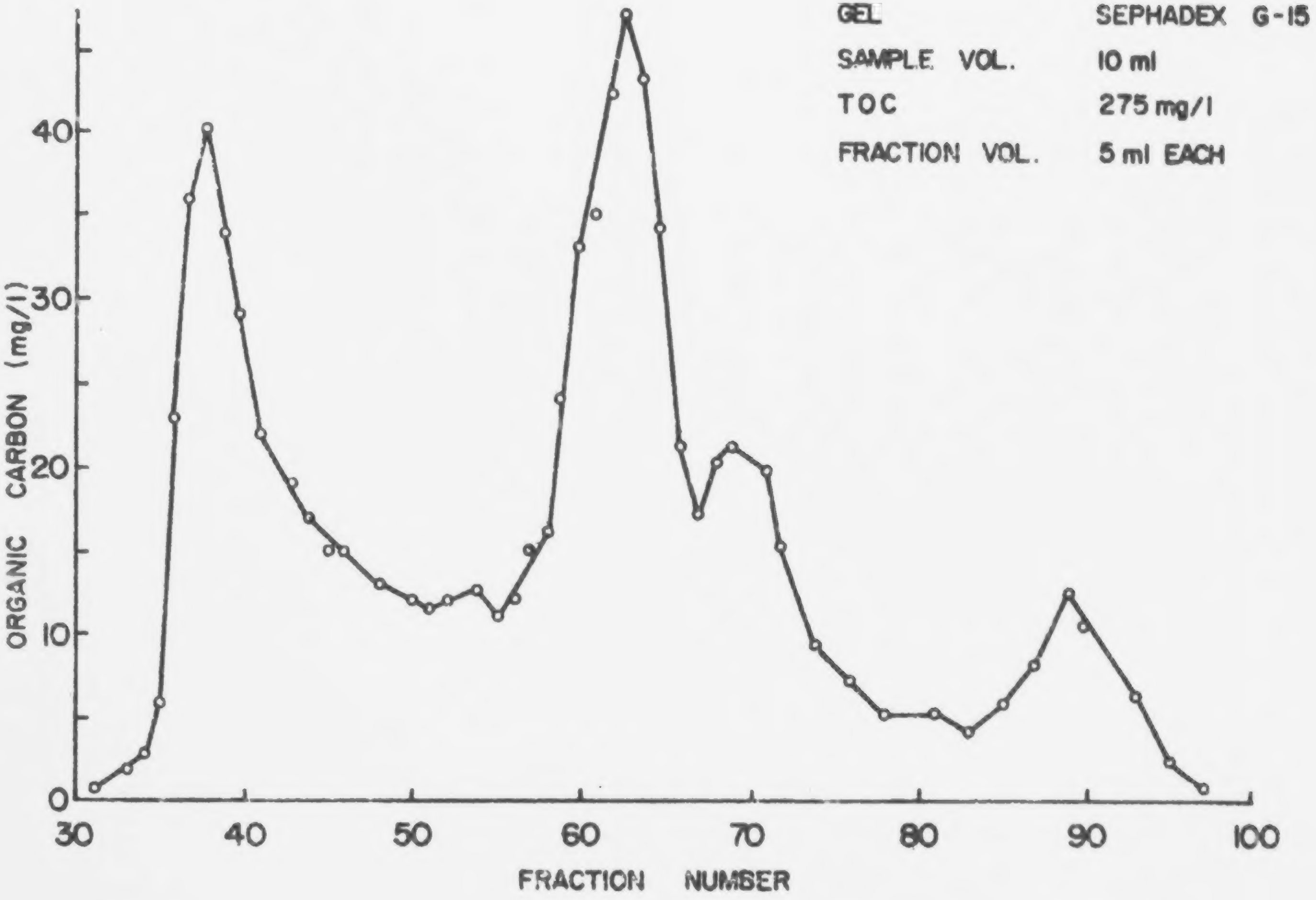

Fig. 6 CHROMATOGRAM OF CLIFTON KNOLLS EFFLUENT FREEZE DRIED CONCENTRATE

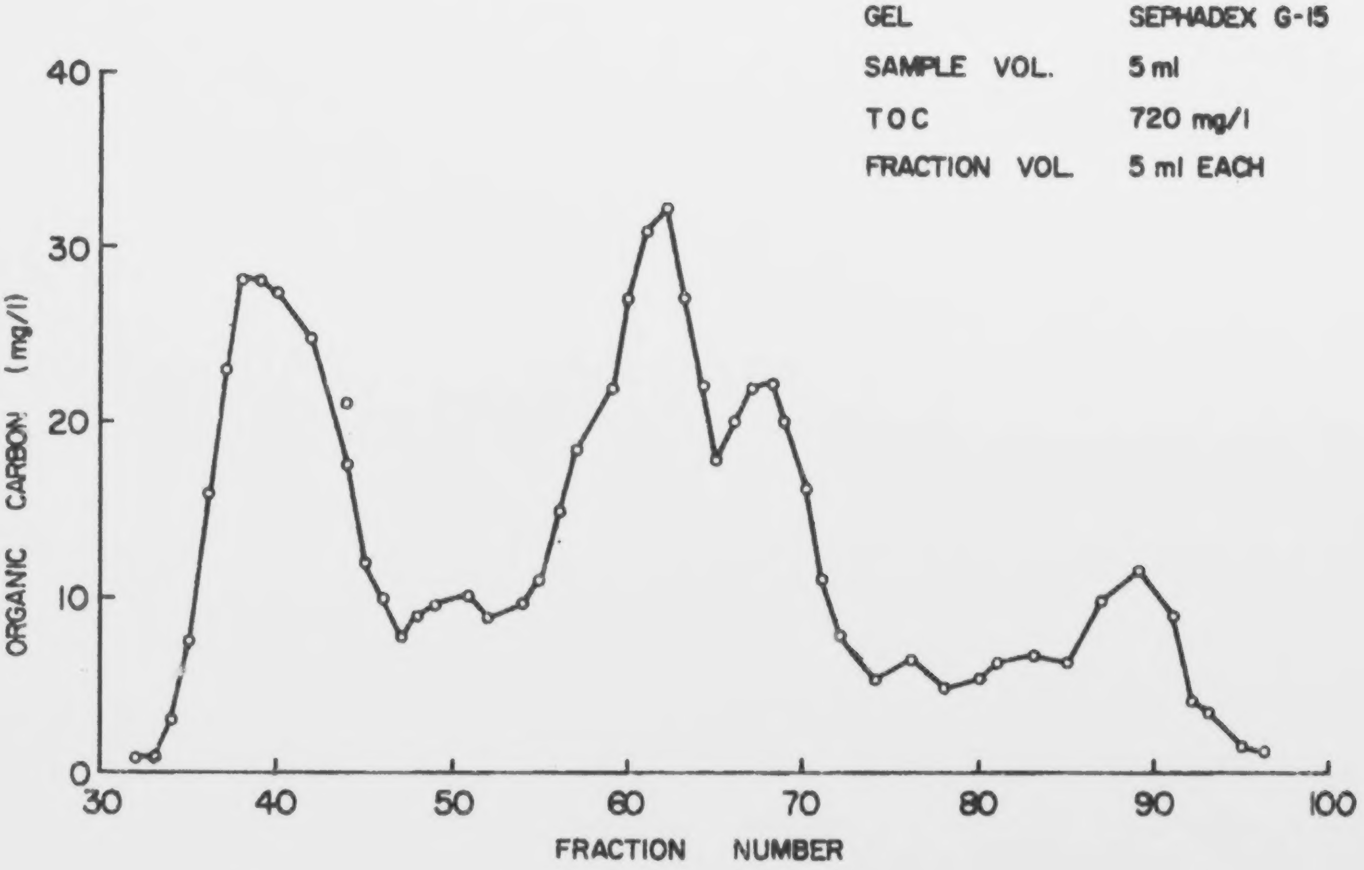

Fig 7 Chromatogram of Clifton Knolls effluent thin film concentrate

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TABLE 5

CHROMATOGRAPHIC DATA FOR CLIFTON KNOLLS EFFLUENT

Preliminary Data	Thin Film Evaporation	Freeze-Drying
Volume of the sample applied	5 ml	10 ml
Volume of gel column	444 cm^3	444 cm^3
Sample volume as percentage of column volume	1.13%	2.25%
Organic content of sample	720 mg/l	275 mg/l
Total organic carbon applied	3.6 mg	2.75 mg
Rate of flow through the column	38 ml/hr	40 ml/hr
Molecular Weight Distribution Data		
Compounds greater than apparent molecular weight 1,500 in the sample	17%	18%
Compounds greater than apparent molecular weight 1,100 in the sample	32%	31%
Compounds greater than apparent molecular weight 500 in the sample	43%	41%
Compounds greater than apparent molecular weight 180 in the sample	73%	74%
Compounds greater than apparent molecular weight 100 in the sample	86%	83%

to its molecular weight and it has come out approximately at the end of the bed volume. No definite molecular weight can, therefore, be assigned to this peak which accounts for about 9% of the organic carbon present in the sample.

All the peaks appear at the same elution volume in both the chromatograms. The percentage curves shown in Figure 8 plot out very close to each other, with the maximum difference at any section less than 3 percent, which is believed to be due to the instrumental and other errors involved. No relative polymerization or hydrolysis is, therefore, observed by either concentration techniques.

Molecular weight distribution in the effluent sample, as revealed by the chromatograms, is such that about 30% of the soluble organic material is larger than molecular weight 1,200 and about 28% are smaller than molecular weight 180.

3. Lake George Water

Concentration. Lake George water was the first to be concentrated. However, this was done only to gain experience about the procedure to be adopted in the research. Five liters of the sample were concentrated to 50 ml by thin film evaporation and 4.5 liters were concentrated by freeze-drying in 9 flasks to a final volume of 58 ml. The concentrates were not filtered nor was any acid filtrate obtained. The organic carbon recovery in freeze-drying was found to be only 65% of that of thin film evaporation (Table 6). This was, however, thought to be mainly due to the discrepancies inherent in the initial work, such as lack of experience, improper attention, and less than optimum operating performance of the apparatus.

Another sample was collected on September 5, 1970 from Smith Bay at Lake George, New York, in a 20 liter carboy. The temperature of the sample was 20.5°C and its pH was 7.7. The sample was filtered and concentration was started simultaneously by the two methods. The rotary evaporator outlet was also connected to the freeze-drying apparatus. The vacuum hose which was previously connected to the water aspirator was now connected to one of the ports of the manifold. The temperature of the water bath was 33°C-35°C. The water evaporating from the sample was being condensed

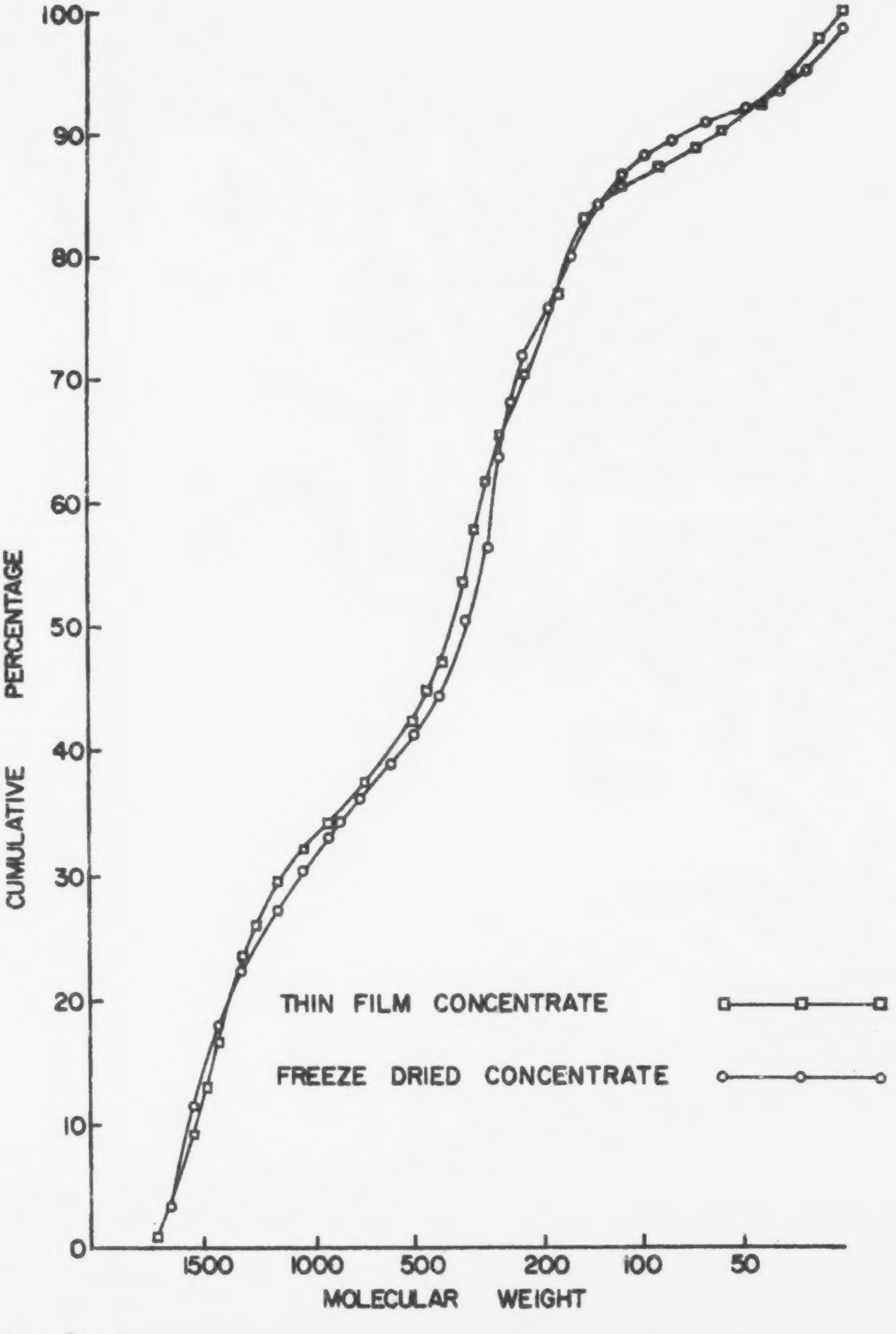

Fig. 8 PERCENTAGE CURVES FOR CHROMATOGRAMS OF CLIFTON KNOLLS EFFLUENT

TABLE 6

CONCENTRATION AND ORGANIC RECOVERY DATA

LAKE GEORGE WATER

SAMPLE NO. 1

Concentration Data	Thin Film Evaporation	Freeze-drying
Initial volume of the sample	5 liters	4.5 liter
Final volume	50 ml	58 ml
Concentration factor	100	77.6
Temperature of water bath	35°C-37°C	-----
Vacuum pressure	40-60 mm of Hg	50-100 u of Hg
Organic Recovery Data		
Organic carbon content of the concentrate	207 mg/l	104 mg/l
Total organic content in the sample (as recovered)	10.35 mg	6.03 mg
Total organic carbon recovered per liter of the sample	2.07 mg	1.34 mg
Percentage recovery (based on 100% recovery in thin film evaporation)	100%	64.8%

into ice in the condenser. The increased temperature gradient and high vacuum of the freeze-dryer resulted in a very rapid concentration of the sample. Two liters were evaporated in less than 16 hours. Such a quick rate of evaporation was not visualized and complete drying of the sample occurred. Five liters of the sample were freeze-dried using 5 flasks (500 ml in each flask, 2 times). The freeze-dried concentrate of this sample also became completely dry. The residues were dissolved with double-distilled water (free of organics) and they were filtered through a membrane filter (0.45 u). The precipitate was dissolved in dilute (1:10) HCl, as per usual procedure and organic carbon in both the concentrate and the acid filtrate was determined on the carbon analyzer. The results show an organic carbon recovery of 87.9% in the freeze-drying method as compared to the thin film evaporation. Concentration data are shown in Table 7.

Chromatographic studies. The flow in the gel column had decreased considerably. The column was, therefore, unpacked and the gel (Sephadex G-15) was washed thoroughly with double-distilled water. The column was then packed with this gel and was again standardized. The calibration curve for the column is shown in Figure 9.

A 10 ml aliquot of each concentrate was applied to the G-15 gel column for separation of the compounds of different molecular weights or sizes. Phosphate buffer was used as eluent (flow rate was 54 ml/hr). The 5 ml fractions collected were analyzed on the total carbon analyzer for organic carbon only. The chromatogram for the thin film concentrate is shown in Figure 10 and that for the freeze-dried concentrate is shown in Figure 11. The readings for individual fractions are given in a later section. Table 8 shown the chromatographic data for the two concentrates.

As in the case of other samples the two chromatograms look alike. There are two major peaks at apparent molecular weight 1,500 and 180 and three minor peaks at apparent molecular weight 600, 370 and 275. There is one very small peak at the end of elution of the sample. All these peaks (even the smallest one) occur in both the chromatograms and essentially at the same elution volume for each peak. About 37% of the compounds are of apparent molecular weight 180 and compounds greater than molecular weight 1,500 constitute about 15%.

TABLE 7

CONCENTRATION AND ORGANIC RECOVERY DATA

LAKE GEORGE WATER

SAMPLE NO. 2

Concentration Data	Thin Film Evaporation	Freeze-drying
Initial volume of the sample	2 liters	5 liters
Final volume (concentrate)	14.5 ml	20 ml
Volume of the acid filtrate	13 ml	10 ml
Concentration factor (based on concentrate volume only)	138	250
Temperature of the water bath	33°C-35°C	-----
Vacuum pressure	40-60 mm of Hg	20-50 u of Hg
Organic Recovery Data		
Organic carbon content		
Concentrate	296	504
Acid filtrate	49	75
Total content in the sample (as recovered)	4.929 mg	10.830 mg
Total organic carbon recovered per liter of the sample	2.465 mg	2.166 mg
Percentage recovery (based on 100% recovery in thin film evaporation)		
Total including acid	100%	87.9%
Concentrate only	100%	93.7%

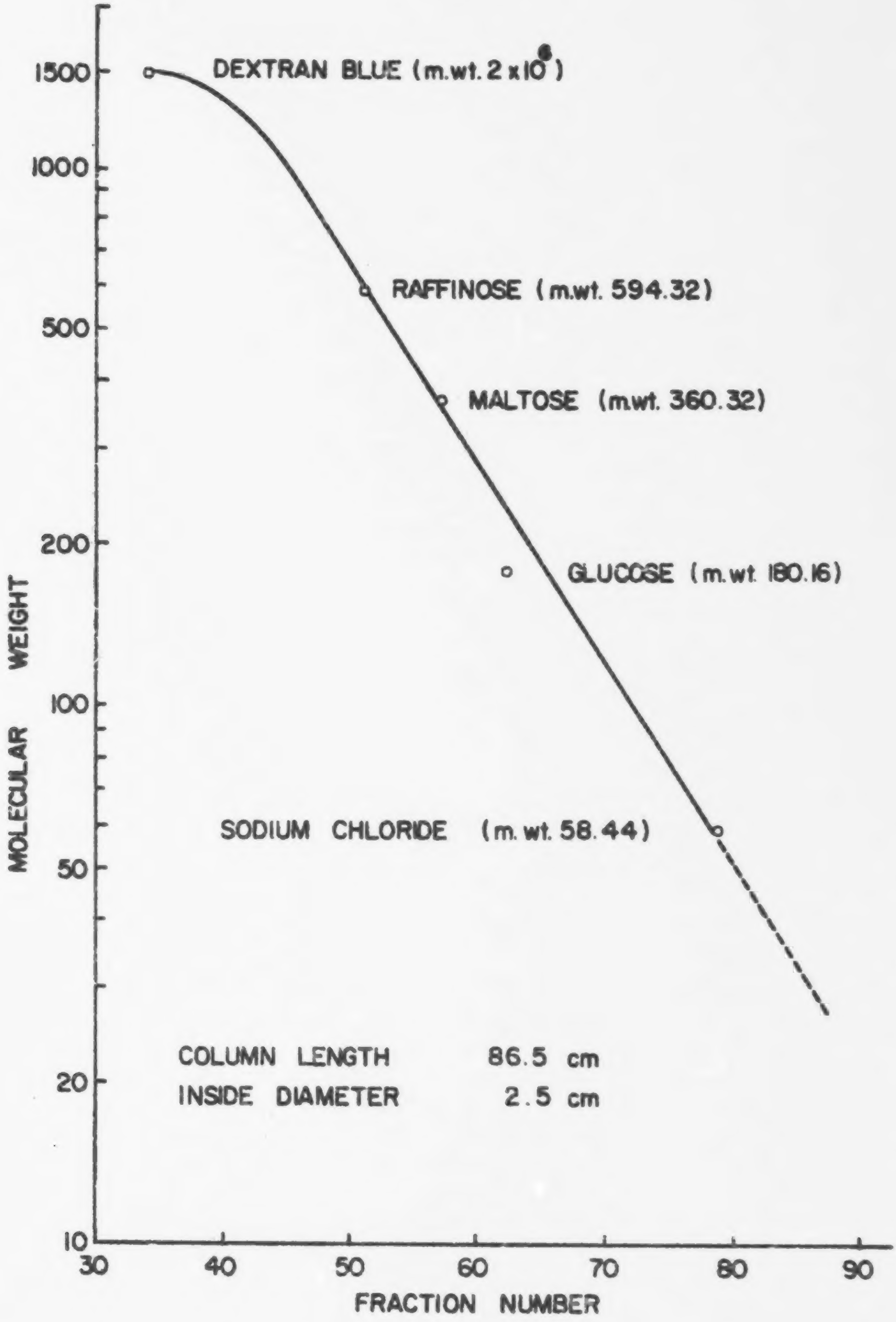

Fig. 9 CALIBRATION CURVE FOR SEPHADEX G-15 COLUMN

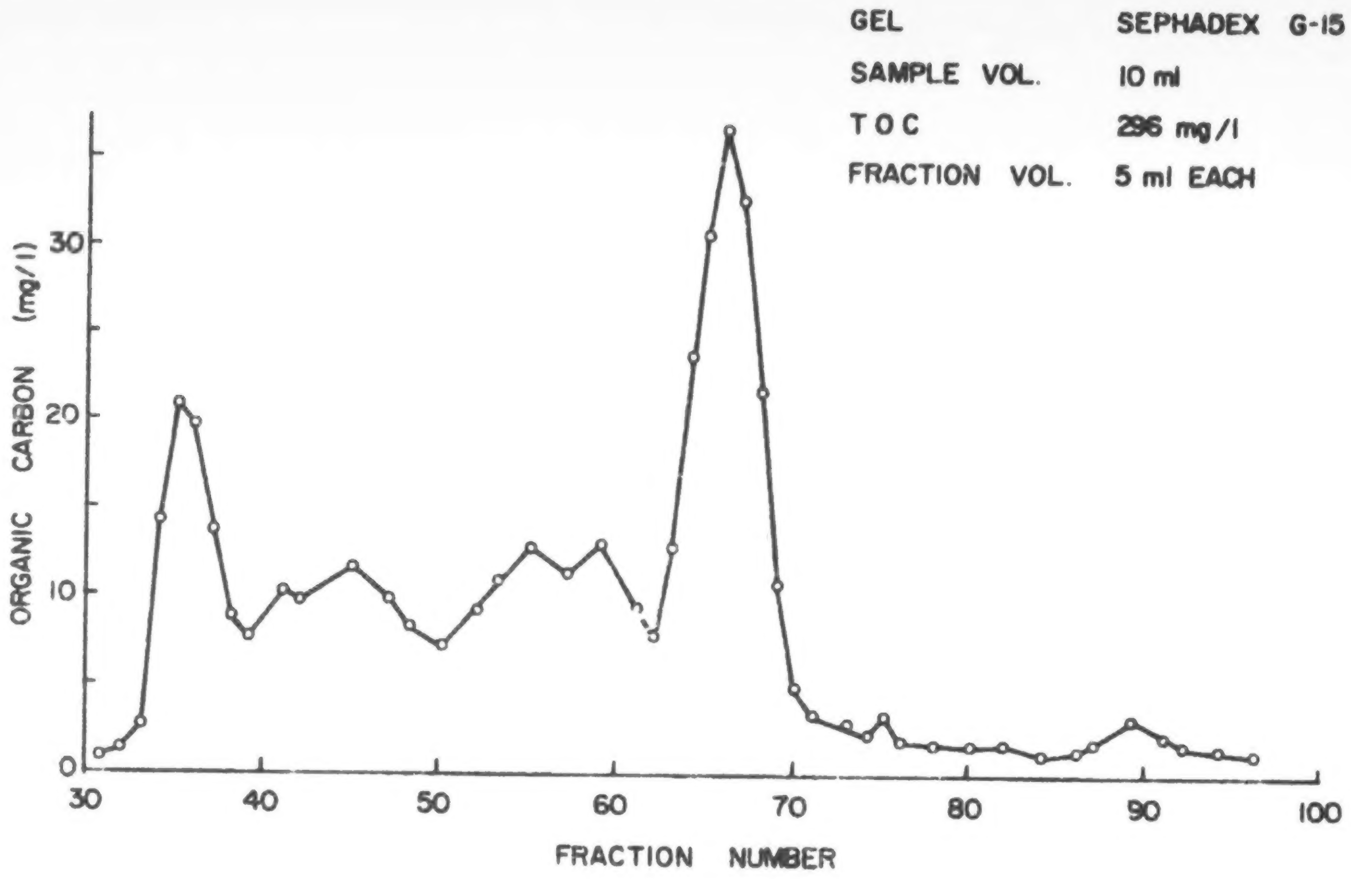

Fig. 10 Chromatogram of Lake George water thin film concentrate

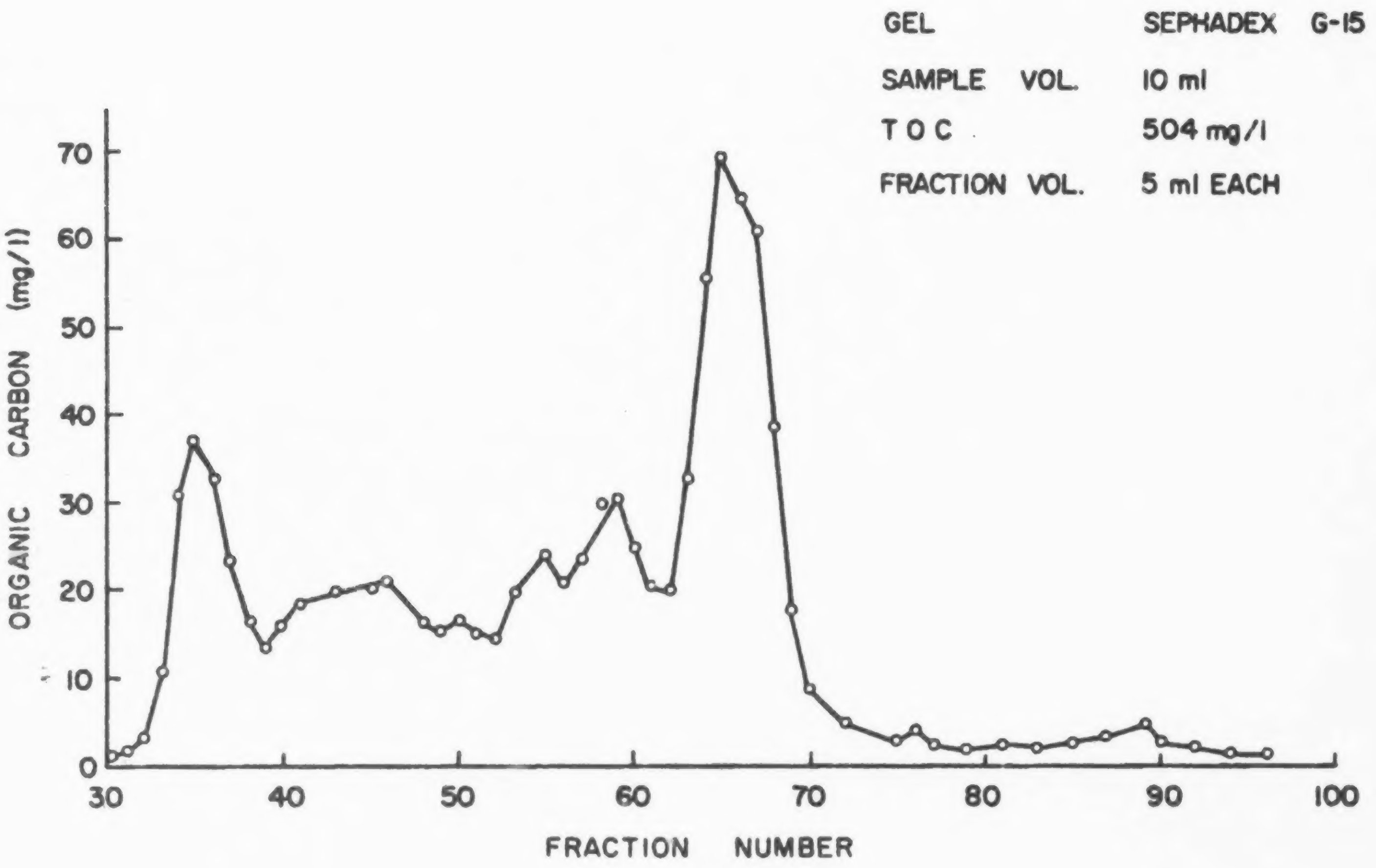

Fig. 11 CHROMATOGRAM OF LAKE GEORGE WATER FREEZE DRIED CONCENTRATE

TABLE 8

CHROMATOGRAPHIC DATA FOR LAKE GEORGE WATER

SAMPLE NO. 2

Preliminary Data	Thin Film Evaporation	Freeze-drying
Volume of the sample applied	10 ml	10 ml
Volume of gel column	425 cm^3	425 cm^3
Sample volume as percentage of column volume	2,35%	2.35%
Organic content of sample	296 mg/l	504 mg/l
Total organic carbon applied to column	2.96 mg	5.04 mg
Rate of flow through the column	54 ml/hr	54 ml/hr
Molecular Weight Distribution Data		
Compounds greater than apparent molecular weight 1,350 in the sample	17%	16%
Compounds greater than apparent molecular weight 600 in the sample	36%	35%
Compounds greater than apparent molecular weight 250 in the sample	61%	61%
Compounds greater than apparent molecular weight 150 in the sample	92%	93%

The percentage curves for the two concentrates, shown in Figure 12, plot out close to each other and do not differ by more than 2% at any section. The difference is, however, too minor to arrive at any definite conclusion. It is believed that instrumental and other errors involved are responsible for this small difference. The possibility of any relative degradation in this case also is, therefore, not apparent.

4. Raw Sewage, Elnora

Concentration. About 5 liters of raw sewage was collected on April 15, 1970 from the sewage treatment plant at Clifton Knolls. The temperature of the sample was 21° C and the pH was 7.7. Five ml of $HgCl_2$ (stock solution with 40 grams of $HgCl_2$/liter) was added to the sample to eliminate microbial activity during storage. The sample was filtered twice, through a coarse filter and a 0.45 u membrane filter.

Five hundred ml of the sample was put in the 2 liter round bottom flask and was concentrated to 53 ml by rotary thin film evaporation. The temperature of the water bath was maintained at 34°-35°C and the vacuum provided with the help of a water aspirator was from 1 to 2 inches of Hg. A small amount of deposit had formed on the flask walls as in the case of Clifton Knolls effluent sample. The deposit was dissolved in dilute HCl at the time of obtaining acid filtrate. Concentration of another 500 ml of the sample was carried out by freeze-drying using only one flask. Final volume of the concentrate was 121 ml. The concentration and organic recovery data for the sample are shown in Table 9.

The original organic content in the sample was quite high, ie 62 mg/l. Therefore, the sample was concentrated only 4 to 9 times. The organic carbon recovery was 92.5% in the case of thin film evaporation and 91.7% in the case of freeze-drying. So, the recovery in freeze-drying for this sample is 99.2% of that in thin film evaporation.

Chromatographic studies. A 10 ml aliquot of each concentrate was applied to G-15 Sephadex column. Phosphate buffer was used as eluent and its flow rate through the column was 48 ml/hr. Five ml fractions obtained were analyzed on the carbon analyzer. Individual readings for each fraction are shown in a later section. The chromatograms obtained are shown in Figure 13 and 14.

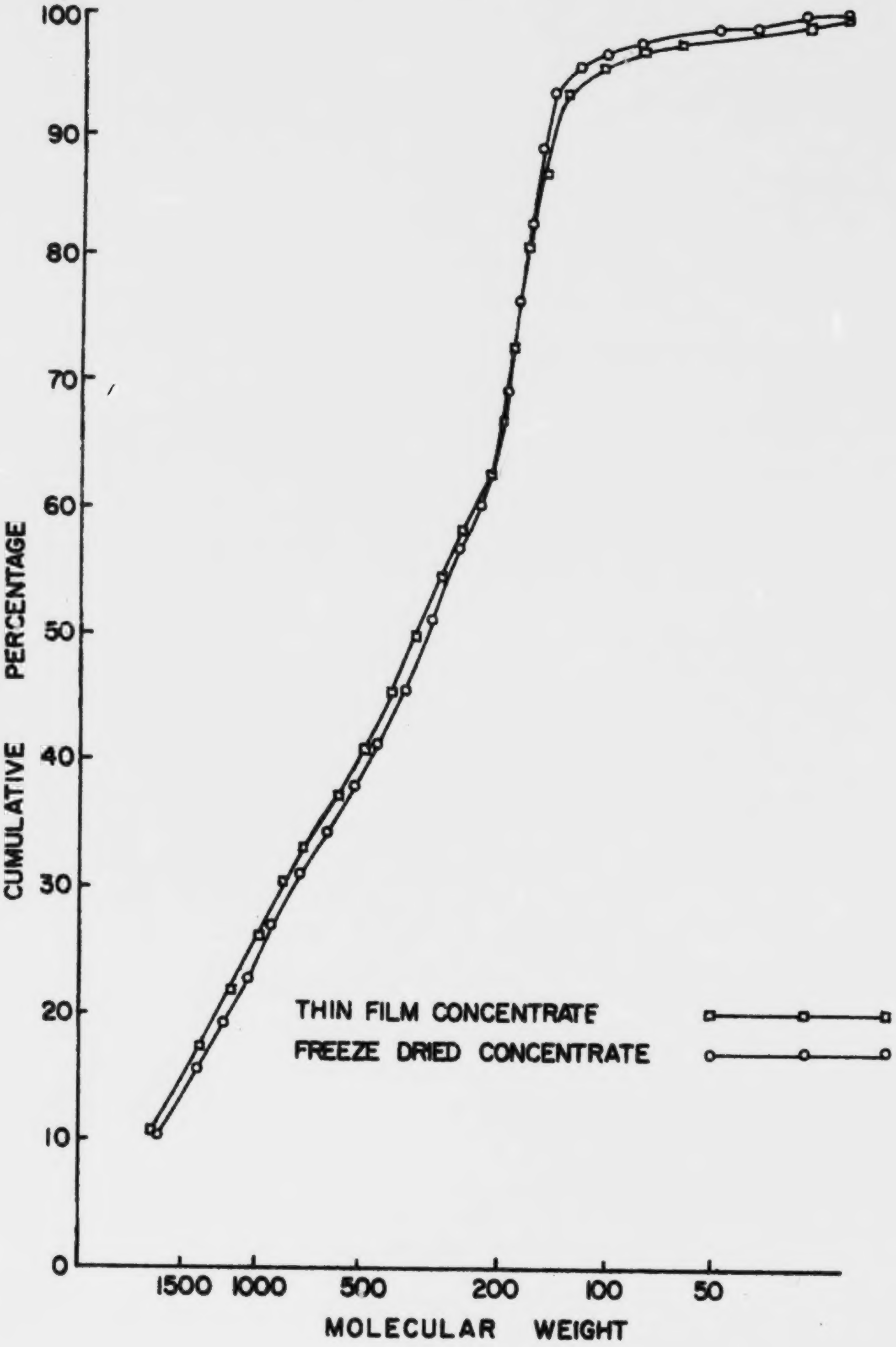

Fig. 12 PERCENTAGE CURVES FOR CHROMATOGRAMS OF LAKE GEORGE WATER

TABLE 9

CONCENTRATION AND ORGANIC RECOVERY DATA

RAW SEWAGE, ELNORA

Concentration Data	Thin Film Evaporation	Freeze-drying
Initial volume of the sample	500 ml	500 ml
Final volume (concentrate)	53 ml	121 ml
Volume of acid filtrate	14 ml	12 ml
Concentration factor (based on concentrate volume only)	9.95	4.1
Temperature of water bath	34^{o}-35^{o}C	-----
Vacuum pressure	40-60 mm of Hg	20-50 u of Hg
Organic Recovery Data		
Organic carbon content		
original sample	62 mg/l	62 mg/l
concentrate	522 mg/l	230 mg/l
acid filtrate	68 mg/l	48 mg/l
Total organic content		
in the sample	31.000 mg	31.000 mg
as recovered	28.618 mg	28.406 mg
Percentage recovery	92.5%	91.7%
Percentage recovery based on 100% recovery in thin film evaporation		
total including acid filtrate	100%	99.2%
concentrate only	100%	100.6%

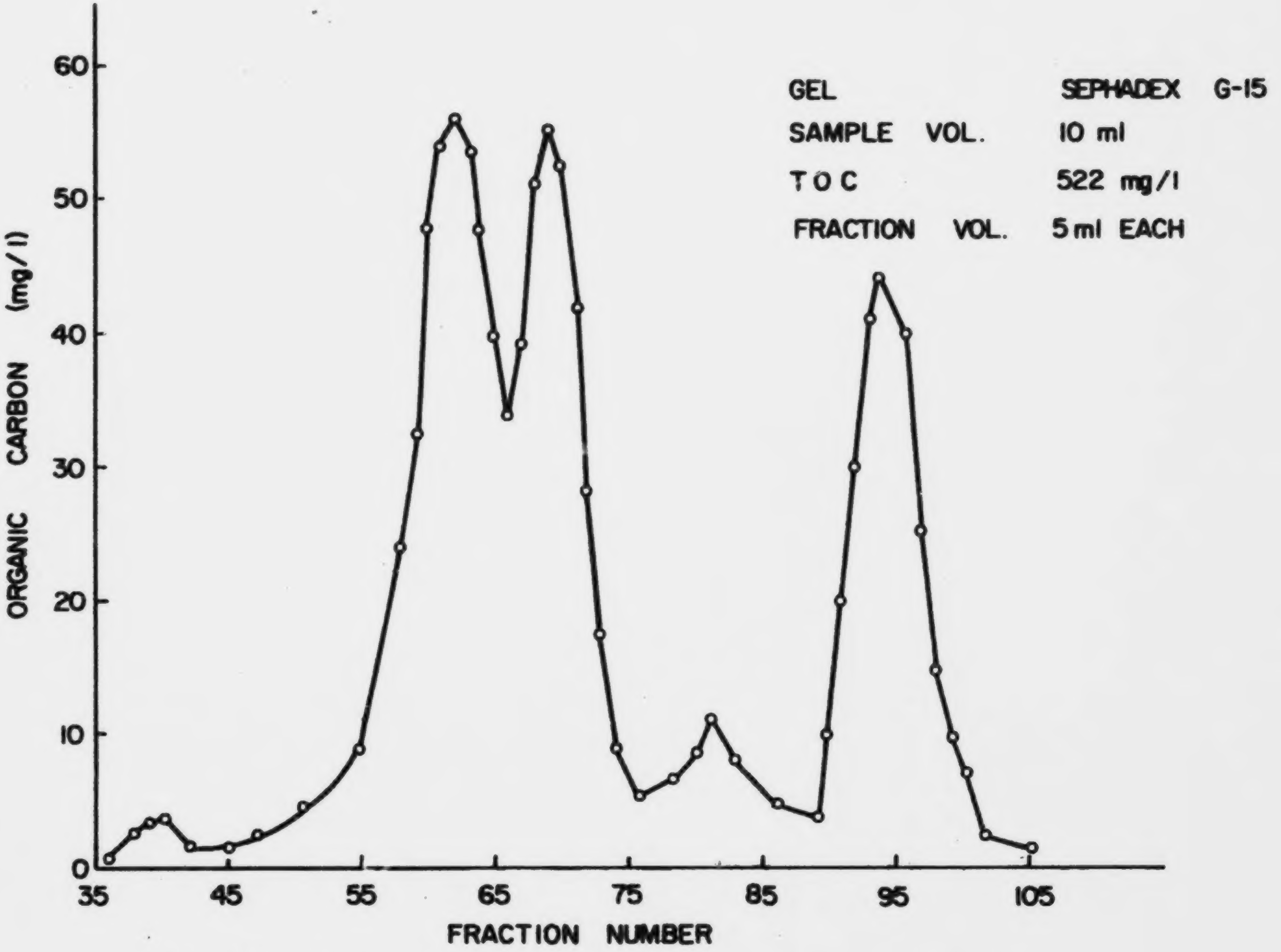

Fig. 13 Chromatogram of Elnora raw sewage thin film concentrate

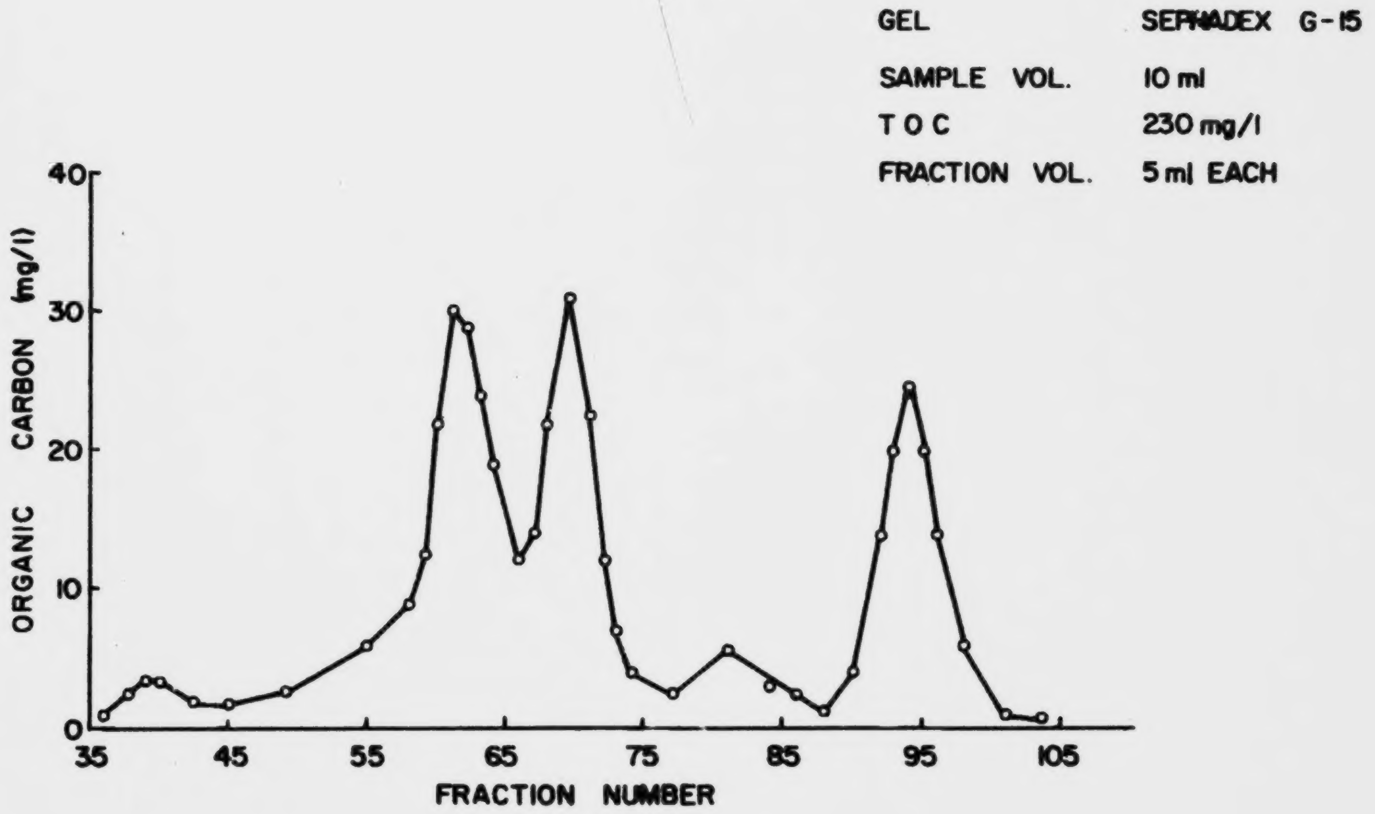

Fig. 14 CHROMATOGRAM OF ELNORA RAW SEWAGE FREEZE DRIED CONCENTRATE

Two major peaks appear in both chromatograms at apparent molecular weight 300 and 180. Another large peak appears at the end of the bed volume. No molecular weight can be assigned to this peak as it cannot be ascertained from the calibration curve, being at the end of the bed volume. This peak accounts for 23% of the organic matter in the sample.

Two minor peaks appear at apparent molecular weight 1,500 and 60.

All the five peaks appear at exactly the same elution volume in both the chromatograms. The percentage curves for the two concentrates (Figure 15) plot out very close to each other with a maximum difference of less than 2% at any section. Obviously, the polymerization or hydrolysis of any compound during concentration by the two methods, has not occurred. Eighty-five percent of the soluble organic compounds are smaller than apparent molecular weight 400 and less than 10% are larger than molecular weight 1,200. The chromatographic data for the sample are shown in Table 10.

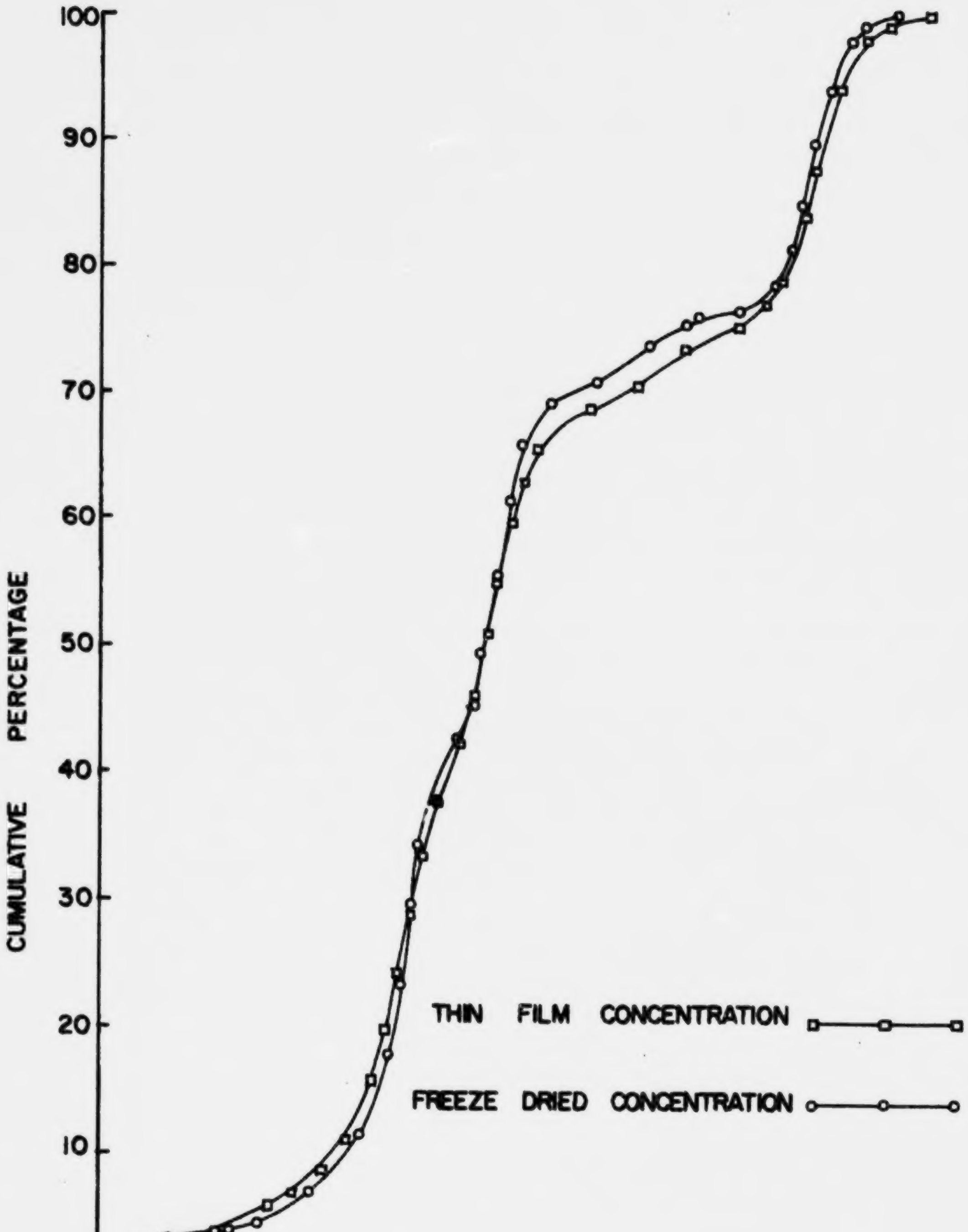

Fig. 15 PERCENTAGE CURVES FOR CHROMATOGRAMS OF ELNORA RAW SEWAGE

TABLE 10

CHROMATOGRAPHIC DATA FOR RAW SEWAGE, ELNORA

Preliminary Data	Thin Film Evaporation	Freeze-drying
Volume of the sample applied	10 ml	10 ml
Volume of gel column	444 cm^3	444 cm^3
Sample volume as percentage of column volume	2.25%	2.25%
Organic content of sample	522 mg/l	230 mg/l
Total organic carbon applied to column	5.22 mg	2.30 mg
Rate of flow through the column	48 ml/hr	48 ml/hr
Molecular Weight Distribution Data		
Compounds greater than apparent molecular weight 1,500 in the sample	2%	2%
Compounds greater than apparent molecular weight 400 in the sample	13%	12%
Compounds greater than apparent molecular weight 200 in the sample	43%	42%
Compounds greater than apparent molecular weight 100 in the sample	68%	70%

SECTION V: DISCUSSION

Two lake samples and two sewage samples were concentrated by thin film evaporation as well as by freeze-drying. In all cases the concentration resulted in a solid and a liquid phase. The liquid phase contained from 70 to 98% of the organic matter recovered. This conforms with the findings of Gjessing and Lee (33) who observed that 60 to 95% of the organics remained in solution on concentration. The amount of organic matter trapped in the solid phase would depend largely on the concentration factor and the nature of the sample.

In both the sewage samples a deposit had formed on the flask walls when these were concentrated by thin film evaporation. There was more deposit in the case of the Clifton Knolls sample because of a greater concentration factor, ie 55 instead of 9.5 in the raw sewage sample. Similarity of the chromatograms obtained in this work, as discussed hereafter, discount the possibility of any interference due to these deposits on the results. This is in accord with Fredette (17) who also observed that deposits on the flask walls did not affect his results.

Gjessing and Lee (33) have observed that color of the samples decreased upon concentration. This is contrary to what has been noticed in this work. Although no attempt was made to quantitatively measure the color before and after concentration, it was observed that the color of the Saratoga Lake water sample and the raw sewage sample had increased on concentration. The increase in color was more pronounced in the case of thin film evaporation than in the case of freeze-drying. In general, no decrease in color was noticed upon concentration of any of the samples.

The concentration and organic recovery data for all the samples show that organic carbon recovery in freeze-drying was less than that in thin film evaporation. Some of the reasons for the lower recovery in freeze-drying may be, a) the absence of flask filters, b) the possible suction of dry solute from the freeze-drying flask, c) the use of increased number of flasks, and d) the high vacuum.

Manufacturers of the freeze-drying apparatus recommend the use of filters on the flasks if the sample is to be dried completely. Filters do help to arrest the flow of solute molecules to some extent. However, when these were used, the frozen sample in the flasks started melting. Presumably the resistance to flow of vapor molecules through the filter is so much that the rate of evaporation is considerably decreased. Heat supplied by the atmosphere to the frozen sample is more than that taken up by the evaporating molecules, therefore, the sample starts melting. The use of these filters was, therefore, abandoned.

In the freeze-drying process the sample is usually shell frozen. As the flask is revolved at about 80 rpm, "pure" ice is formed along the walls of the flask. The solute particles remain in solution and are frozen in the end in the concentrated form. These are the first to be exposed to the actions of freeze-drying. Therefore, an increasing shell of dried material develops as the drying boundary progresses into the sample. All water vapor produced by sublimation at the drying boundary must pass by diffusion through this barrier. It is possible that some of the dried material is drawn into the condenser along with the water vapor.

In thin film evaporation only one flask is used to evaporate the whole sample. In freeze-drying, on the other hand, a number of flasks are used depending upon the size of the sample and capacity of the freeze-dryer. The concentrate from these flasks is transferred into one or more flasks which usually need further concentration. Although effort is made to take out all the concentrate without diluting it excessively with rinse water, some of the concentrate may still be lost in the flasks. Moreover, the greater the number of flasks used, the greater may be the amount of dry solute drawn into the condenser.

Absolute pressure in the freeze-drying process is of the order of a few microns, whereas in the thin film evaporation it is in the range of 1-2 inches of Hg. A higher vacuum may contribute to the removal of dry solute from the freeze-drying flasks.

Many organics are only sparingly soluble in water and their concentration could exceed the solubility limit, thereby placing some of the compounds in the precipitate. These might have been difficult to dissolve even in dilute HCl.

Concentration achieved in freeze-drying was many times more than that in thin film evaporation in all of the samples except raw sewage sample. This might have contributed something towards the low recovery in the freeze-drying process.

It is also observed that relative recovery in freeze-drying is better for sewage samples than for water samples. Possibly the recovery in freeze-drying is dependent, to some extent, on the amount of organic matter present in the samples. In low organic water samples comparatively larger portions of dry solute may be lost into the condenser.

The main purpose of this research was to determine if any polymerization of hydrolysis occurred in either of the concentration techniques employed. Gel chromatography was the only convenient way of assessing the relative polymerization or hydrolysis effects. This was carried out in the cold room at 4°C.

The chromatograms obtained show a different molecular weight distribution for each sample. While the Saratoga Lake water sample showed a predominance of high molecular weight compounds in it, the Lake George water sample contained a balanced amount of high and low molecular weight compounds. In the case of Saratoga Lake water, more than 60% of the compounds were of molecular weight greater than 700. This observation is largely in conformity with that of Gjessing and Lee (33) who separated several river water samples of gel columns. They also found a preponderance of large molecular weight (700+) compounds in river waters. The Lake George water which is oligotrophic and unpolluted, had 36% of the compounds larger than molecular weight 600 and 37% of the compounds were with molecular weight about 180.

Chromatograms for sewage samples indicate that the raw sewage sample had 85% of the compounds smaller than molecular weight 400. The effluent sample, on the other hand, had only 42% of the compounds smaller than molecular weight 400. This tends to indicate that low molecular weight compounds in raw sewage are comparatively easily removed by biological treatment.

As freeze-drying is carried out at much lower temperatures, one would expect some difference in the chromatograms for thin film and freeze-dried concentrates. Absence of a peak in one of the chromatograms would mean that the compound which was to come out at the elution volume had either been altered, polymerized, or hydrolyzed, or has been totally removed by the means employed for concentration. No meaningful difference in the two chromatograms has, however, been found for the samples analyzed in this investigation.

In all cases the volume of the sample applied to the gel column was between 1% and 3% of the bed volume and the rate of flow through the column varied from 28 ml/hr to 54 ml/hr. Two chromatograms obtained for the thin film and freeze-dried concentrates of the same sample have an identical number of peaks and at about the same elution volume, indicating, therefore, the presence of the same compounds in both concentrates. The percentage curves for the two chromatograms which indicate the percentage of compounds larger than a certain molecular weight, do not show a difference of more than 2 to 3% for any molecular weight. This minor difference is considered to be due to instrumental and other errors involved. McDonald et al (22) have reported that chromatograms for unconcentrated raw sewage and raw sewage concentrated by freeze-drying looked identical, showing no alteration of the compounds when concentrated by freeze-drying. It is, therefore, concluded that no polymerization or hydrolysis occurred during concentration by either method.

It appears that not many heat labile compounds were present in the water and wastewater samples analyzed, and if these were present, the heat supplied in the thin film evaporation was not sufficient to effect any degradation of the compounds. The Lake George water sample was also concentrated at 60°C by thin film evaporation. The chromatogram obtained was identical to the one obtained for the sample concentrated at 35°C showing, thereby, the absence of heat labile compounds.

It is also believed that highly volatile compounds, if present, must have been removed nearly equally in both methods. The possibility of selective removal of a compound in either method is discounted because of the similarity of the two chromatograms.

The main drawback in thin film evaporation is the excessive time required to concentrate the sample. On the average, one liter of sample could be concentrated in 24 hours. The rate of evaporation can, however, be increased drastically if the apparatus is connected to a suitable vacuum pump and condenser.

As stated above, the low recovery in the freeze-drying method is mainly due to the suction of dry solute into the condenser. The loss could be minimized with the help of a suitable device such as an improved filter which would exclude the dry solute matter without hindering the transfer of vapor molecules. From the results of this research, however, it may be concluded that it would be appropriate to use thin film evaporation for concentrating water samples and freeze-drying for concentrating sewage samples.

Freeze-drying has the inherent advantage, over thin film evaporation of being carried out at much lower temperatures, which affords less chance for degradation of the sample. If a sewage sample cannot be preserved (it was preserved in this research with $HgCl_2$) due to the special nature of the experiment, freeze-drying might be the only reasonable way of concentrating the unpreserved sample.

The possibility of carrying out thin film evaporation at reduced temperatures, ie less than 10°C, needs to be investigated. This would combine the advantages of both freeze-drying and thin film evaporation, ie less chance for degradation and greater organic carbon recovery, respectively. Lowering the temperature is certain to reduce the rate of evaporation, but connecting the apparatus to a vacuum pump and a condenser may make up for most of the loss.

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SECTION VII: ORGANIC CARBON OF FRACTIONS FROM SEPHADEX G-15 COLUMN

Saratoga Lake Water Concentrated
By Thin Film Evaporation

Sample Volume 5 ml
T.O.C. 100 mg/1

Fraction No.	Organic Carbon mg/1	Fraction No.	Organic Carbon mg/1
31	1.0	64	3.0
32	1.0	65	2.5
33	1.5	66	1.5
34	2.0	67	1.5
35	5.5	68	2.0
36	7.5	69	2.2
37	8.2	70	1.5
38	8.5	71	1.5
39	8.0	72	1.3
40	10.0	73	2.0
41	11.0	74	2.0
42	12.0	75	1.0
43	11.5	76	2.0
44	10.0	77	1.5
45	9.5	78	2.0
46	9.0	79	1.0
47	8.5	80	1.5
48	7.0	81	1.5
49	6.5	82	1.5
50	5.0	83	1.5
51	5.0	84	1.0
52	5.5	85	1.5
53	6.0	86	2.0
54	7.0	87	1.0
55	9.0	88	2.0
56	9.5	89	1.5
57	7.2	90	2.0
58	7.3	91	1.0
59	6.5	92	1.5
60	6.0	93	0.6
61	5.5	94	1.0
62	6.5	95	1.0
63	4.0	96	0.5

ORGANIC CARBON OF FRACTIONS FROM SEPHADEX G-15 COLUMN

Saratoga Lake Water Concentrated
By Freeze-Drying

Sample Volume 10 ml
T.O.C. 100 mg/l

Fraction No.	Organic Carbon mg/l	Fraction No.	Organic Carbon mg/l
32	1.0	62	4.3
33	2.0	63	3.8
34	4.0	64	3.4
35	5.0	65	2.3
36	6.0	66	1.8
37	6.5	67	1.9
38	7.0	68	2.0
39	6.5	69	1.9
40	9.0	70	2.0
41	9.5	71	3.0
42	10.0	72	2.6
43	9.0	73	2.6
44	9.0	74	2.5
45	8.5	75	2.5
46	8.2	76	1.8
47	8.0	77	1.6
48	5.9	78	1.6
49	5.4	79	1.5
50	4.5	80	1.2
51	4.3	81	1.1
52	4.4	82	1.4
53	5.1	83	1.7
54	5.7	84	1.7
55	6.6	85	1.8
56	6.9	86	1.6
57	6.0	87	1.5
58	5.8	88	1.6
59	5.5	89	2.0
60	5.0	90	1.5
61	5.4	91	1.6

ORGANIC CARBON OF FRACTIONS FROM SEPHADEX G-15 COLUMN

Clifton Knolls Effluent Concentrated
By Thin Film Evaporation

Sample Volume 5 ml
T.O.C. 720 mg/l

Fraction No.	Organic Carbon mg/l	Fraction No.	Organic Carbon mg/l
31	1.0	64	22.0
32	1.0	65	18.0
33	1.5	66	20.0
34	3.0	67	22.0
35	7.5	68	22.0
36	16.0	69	20.0
37	23.0	70	16.0
38	28.0	71	11.0
39	28.0	72	8.0
40	27.5	73	6.0
41	29.0	74	5.5
42	25.0	75	6.0
43	20.0	76	6.5
44	17.0	77	6.0
45	12.0	78	5.0
46	10.0	79	5.0
47	8.0	80	5.5
48	9.0	81	6.5
49	9.5	82	6.6
50	8.5	83	6.7
51	10.0	84	6.5
52	9.0	85	6.5
53	9.5	86	8.0
54	10.0	87	10.0
55	11.0	88	11.0
56	15.0	89	11.5
57	18.5	90	10.0
58	20.0	91	9.0
59	22.0	92	4.0
60	27.5	93	3.5
61	31.0	94	1.7
62	32.0	95	1.5
63	27.0	96	1.0

ORGANIC CARBON OF FRACTIONS FROM SEPHADEX G-15 COLUMN

Clifton Knolls Effluent Concentrated
By Freeze-Drying

Sample Volume 10 ml
T.O.C. 275 mg/l

Fraction No.	Organic Carbon mg/l	Fraction No.	Organic Carbon mg/l
31	2.0	66	22.0
32	2.5	67	18.0
33	3.0	68	21.0
34	4.0	69	22.0
35	7.0	70	21.5
36	24.0	71	20.5
37	37.0	72	16.0
38	41.0	73	12.0
39	35.0	74	10.0
40	30.0	75	9.0
41	23.0	76	8.0
42	22.0	77	7.0
43	20.0	78	6.0
44	18.0	79	6.0
45	16.0	80	6.0
46	16.0	81	6.0
47	15.0	82	5.0
48	14.0	83	5.0
49	13.0	84	6.0
50	13.0	85	6.5
51	12.5	86	7.0
52	13.0	87	9.0
53	13.5	88	11.0
54	13.5	89	13.0
55	12.0	90	11.0
56	13.0	91	10.5
57	16.0	92	9.0
58	17.0	93	7.0
59	25.0	94	4.0
60	34.0	95	3.0
61	36.0	96	2.0
62	43.0	97	1.5
63	48.0	98	1.5
64	44.0	99	1.0
65	35.0	100	1.5

ORGANIC CARBON OF FRACTIONS FROM SEPHADEX G-15 COLUMN

Lake George Water Concentrated
By Thin Film Evaporation

Sample Volume 5 ml
T.O.C. 296 mg/l

Fraction No.	Organic Carbon mg/l	Fraction No.	Organic Carbon mg/l
30	1.5	64	24.0
31	1.5	65	31.0
32	1.5	66	37.0
33	3.0	67	33.0
34	14.5	68	22.0
35	21.0	69	11.0
36	20.0	70	5.0
37	14.0	71	3.5
38	9.0	72	3.0
39	8.0	73	3.0
40	9.0	74	2.5
41	10.5	75	3.5
42	10.0	76	2.0
43	11.0	77	2.0
44	11.5	78	1.7
45	12.0	79	2.0
46	10.5	80	1.8
47	10.0	81	2.0
48	8.5	82	1.7
49	8.0	83	1.3
50	7.5	84	1.2
51	8.0	85	1.3
52	9.5	86	1.4
53	11.0	87	1.8
54	12.0	88	2.3
55	13.0	89	3.3
56	12.0	90	2.4
57	11.5	91	2.4
58	12.5	92	1.8
59	13.0	93	1.9
60	12.0	94	1.5
61	9.5	95	2.1
62	8.0	96	1.3
63	13.0		

ORGANIC CARBON OF FRACTIONS FROM SEPHADEX G-15 COLUMN

Lake George Water Concentrated
By Freeze-Drying

Sample Volume 10 ml
T.O.C. 504 mg/1

Fraction No.	Organic Carbon mg/1	Fraction No.	Organic Carbon mg/1
30	1.5	64	56.0
31	1.5	65	70.0
32	3.0	66	65.0
33	11.0	67	61.0
34	31.0	68	39.0
35	37.0	69	18.0
36	33.0	70	9.0
37	23.0	71	6.0
38	16.5	72	5.0
39	14.0	73	4.0
40	16.0	74	3.5
41	18.5	75	3.0
42	19.5	76	3.5
43	20.0	77	2.5
44	20.0	78	2.0
45	20.5	79	2.0
46	21.0	80	2.2
47	19.0	81	2.5
48	16.5	82	2.3
49	15.5	83	2.1
50	16.5	84	2.0
51	15.0	85	2.5
52	15.0	86	2.6
53	21.0	87	3.5
54	22.0	88	3.7
55	24.0	89	4.0
56	21.0	90	3.0
57	24.0	91	3.0
58	30.0	92	2.0
59	30.5	93	2.0
60	25.0	94	1.7
61	20.5	95	1.5
62	20.0	96	1.7
63	33.0		

ORGANIC CARBON OF FRACTIONS FROM SEPHADEX G-15 COLUMN

Elnora Raw Sewage Concentrated
By Thin Film Evaporation

Sample Volume 10 ml
T.O.C. 522 mg/l

Fraction No.	Organic Carbon mg/l	Fraction No.	Organic Carbon mg/l
33	2.0	71	41.0
34	2.0	72	29.0
35	2.0	73	19.0
36	3.0	74	11.0
37	4.5	75	8.0
38	6.0	76	7.0
39	7.0	77	7.5
40	7.2	78	7.5
41	6.0	79	8.0
42	5.5	80	8.5
43	5.5	81	11.5
44	5.0	82	9.5
45	5.0	83	8.5
46	6.0	84	7.0
47	6.0	85	7.5
48	6.5	86	7.0
49	7.0	87	6.5
50	8.0	88	5.0
51	8.0	89	6.5
52	8.5	90	12.0
53	9.0	91	21.0
54	10.5	92	32.0
55	12.0	93	40.0
56	15.5	94	43.0
57	18.0	95	42.0
58	25.0	96	37.0
59	33.5	97	20.5
60	45.0	98	17.5
61	53.0	99	11.0
62	57.0	100	7.0
63	52.5	101	5.0
64	45.0	102	4.0
65	39.5	103	4.0

ORGANIC CARBON OF FRACTIONS FROM SEPHADEX G-15 COLUMN
(Continued)

Fraction No.	Organic Carbon mg/l	Fraction No.	Organic Carbon mg/l
66	32.0	104	3.0
67	39.0	105	3.0
68	49.5	106	2.5
69	55.5	107	2.0
70	51.5	108	2.0

ORGANIC CARBON OF FRACTIONS FROM SEPHADEX G-15 COLUMN

Elnora Raw Sewage Concentrated
By Freeze-Drying

Sample Volume 10 ml
T.O.C. 230 mg/l

Fraction No.	Organic Carbon mg/l
35	1.0
36	1.0
37	2.0
38	2.5
39	4.7
40	3.5
41	3.0
42	2.5
43	2.0
44	2.0
45	1.5
46	1.5
47	2.0
48	2.0
49	2.5
50	3.0
51	3.5
52	3.5
53	3.5
54	5.0
55	6.0
56	6.0
57	7.0
58	9.0
59	10.0
60	22.0
61	30.0
62	29.5
63	24.0
64	19.0
65	13.0
66	12.0
67	14.0
68	22.0
69	31.0
70	31.5
71	22.5
72	12.0
73	7.0
74	4.0
75	3.0
76	3.0
77	2.5
78	3.0
79	4.0
80	4.5
81	3.5
82	4.5
83	3.5
84	3.0
85	3.0
86	2.0
87	2.0
88	1.5
89	2.0
90	4.0
91	8.0
92	14.0
93	20.0
94	24.0
95	20.0
96	14.0
97	10.0
98	6.0
99	3.0
100	2.0
101	1.0
102	1.0
103	1.0
104	1.0
105	1.0

APPENDIX B

THE EFFECT OF CHEMICAL-PHYSICAL TREATMENT ON THE SOLUBLE ORGANIC COMPONENT OF WASTEWATER

SECTION I: CONCLUSIONS

The work described in this appendix shows that the soluble organic components of various municipal wastewaters all exhibit strikingly similar chromatographic characteristics. Several other conclusions result from this study.

1. The constituents of the soluble organic component of municipal wastewater are primarily of low apparent molecular weight (less than 1,500).

2. High pH lime treatment has no dramatic effect on the apparent molecular weight spectra of the soluble organic component in municipal wastewater.

3. The minimal changes observed in the apparent molecular weight spectra of municipal wastewater after high pH lime treatment can be attributed to some unknown removal mechanism or a combination thereof.

4. The soluble organic component of wastewaters after chemical treatment and activated carbon adsorption is characterized by a predominance of low apparent molecular weight constituents.

5. The freeze-drying technique is an excellent method of concentrating wastewater soluble organics.

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SECTION II: RECOMMENDATIONS

Based on the analysis of the results and experience gained, the following recommendations are made:

1. Further investigations should be made on the identification of the constituents of the soluble organic component in municipal wastewater.

2. Further investigations should be made on the applicability of the gel chromatography technique in designing and controlling wastewater treatment processes.

3. Further investigations should be made on the specificity of activated carbon adsorbtion for soluble wastewater organics in various apparent molecular weight ranges.

4. Investigations of various wastewater treatment schemes aimed at optimizing removals of soluble wastewater organics should be made.

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SECTION III: INTRODUCTION, HISTORY AND TECHNICAL REVIEW

Present conventional wastewater treatment processes are effective in removing particulate organic matter but less effective in removing soluble organic matter. The need for higher quality effluents has spurred research directed toward development of waste treatment processes which are highly efficient in the removal of soluble organic matter.

Reports (6, 40, 42, 49) to date indicate that chemical treatment followed by granular activated carbon adsorption (chemical-physical treatment) is an effective advanced wastewater treatment process and that the molecular size of the soluble organic compounds in wastewater may effect the efficiency of activated carbon adsorption (28, 50, 51). Molof and Zuckerman (54, 55) contend that molecular size is the primary factor affecting adsorption and that the chemical-physical treatment process can be optimized via high lime ($CaOH_2$) dosages. The resulting high pH allegedly breaks down large molecular weight organic compounds by alkaline hydrolysis into their more adsorbable components.

In view of the possible affects of molecular size on adsorption, a study was undertaken to characterize the soluble organic component of wastewater by molecular size and to determine if lime treatment has an effect on that component. A second objective was to obtain data to characterize the soluble organic component after activated carbon adsorption.

Gel chromatography has been used in recent studies (15, 25) to analyze the soluble organic wastewater component. This analytical technique has been particularly suitable for this study since it allows the separation or fractionation of a mixture of water soluble molecules into their approximate molecular sizes. The method also lends itself to the approximate determination of molecular weights of the fractionated compounds after appropriate calibration curves have been developed. Characteristic molecular size spectra or chromatograms can be obtained from samples taken at various points in a treatment process and compared to determine if certain molecular sizes are preferentially removed, broken down or added to the original mixture of solute molecules.

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In view of the applicability of gel chromatography it was decided to use this method and the supporting methods of freeze-dry concentration and combustion infrared total organic carbon determination to accomplish the aims of this study.

The results were obtained in three parts as follows:

Analysis of untreated sewage samples and laboratory lime treated sewage samples to characterize the soluble organic component of sewage and to determine if lime treatment had an effect on it.

Analysis of waste samples from independent chemical-physical treatment pilot plants to further confirm data obtained in Part I and to characterize the soluble organic component after activated carbon adsorption.

Confirmation of the efficacy of the freeze-drying technique and comparison of the effect, on the soluble organic component, of sodium hydroxide as opposed to lime.

Definition and Organic Content of the Soluble Component of Domestic Sewage

A knowledge of how the soluble organic component of sewage is defined and information on its specific organic content was necessary to insure proper techniques and to interpret the data obtained.

1. Definition of the Soluble Organic Component

A classification system based on both size and settling characteristics of sewage fractions was presented by Rudolfs and Gehm (38) in 1939. This classification system consisted of four fractions as follows:

Settleable Solids
Pseudo Colloidal Sols
True Colloidal Sols
Soluble Solids

The true colloidal fraction was defined as those solids passing a 0.2 micron filter and retained on a ultra-filter passing only true solutions.

Rudolfs and Balmat (37) expanded and revised this earlier work to reflect specific size ranges and investigated methods of separating sewage into the various fractions. The electron microscope was used in determining the size ranges obtained by the separation methods investigated. Table 1 summarizes the resulting classification system and separation methods.

Heukelekian and Balmat (16) separated domestic sewage fractions utilizing the classification system outlined in Table 1. Composite winter and summer samples were obtained from a single sewage treatment facility. The samples were then classified and the relative contribution of each fraction to total solids and organic matter was determined. Hunter and Heukelekian (17) reported on a similar study involving the same sewage and procedures used by Heukelekian and Balmat. The contributions of the various fractions to total solids, organic matter and nitrogenous matter were presented. Additionally, Rickert and Hunter (35) modified the separation method by substituting centrifugation at 50,000 rpm for the membrane filtration step. The electron microscope was again used to check the particle size range obtained. The contributions of total solids and organic matter of the fractions were reported. Painter and Viney (31) separated and characterized the fractions of English domestic sewage with respect to organic matter and nitrogenous matter. The fractions were obtained using separation methods comparable with previous work. A summary of the general characteristics of the sewage fractions as derived from the literature is presented in Table 2. Based upon this literature a general similarity in the contribution of each fraction to total organic and nitrogenous matter is indicated.

2. Organic Content of the Soluble Component

Very little data on specific identification of organic groups or compounds could be located in the literature.

Hunter and Heukelekian (17) reported on the ether soluble and ether insoluble matter in the soluble component. The specific compounds identified represented a small part (less than 10%) of the total soluble organic matter and only alkyl benzene sulfonate, sugars, and amino acids were significant contributors.

Table 1. Classification of Sewage Fractions as Presented by Rudolfs and Balmat

Fraction	Size Range	Separation Method
Settleable Solids	greater than 100 microns	settling for 1 hour
Supra Colloidal Solids	1 to 100 microns	continuous centrifuging at 14,000 rpm at 100 cc/hr
Colloidal Solids	1 micron to 1 millimicron	ultra filtration
Soluble Solids	less than 1 millimicron	ultra filtration

Table 2. Typical Strength Distributions of Organic and Nitrogenous Matter in Sewage Fractions as Percent of Total Parameter

	* % Organic Matter			% Nitrogenous Matter		
Fraction	Heukelekian Balmat (16)	Hunter Heukelekian (17)	Rickert Hunter (35)	Painter and Viney (31)	Hunter Heukelekian (17)	Painter and Viney (31)
Settleable	31.5	30	24	34	23	23
Supracolloidal	23.5	19	18.5	22	34	20
Colloidal	14	10	9	15	11	20
Soluble	31	41	48.5	29	22	37

* As determined by volatile suspended solids or organic carbon

Painter and Viney (31) and Painter, Viney and Bywaters (32) reported on a complete study of the soluble component. Of particular significance to this study is the fact that these workers used freeze-dry concentration and measured organics as organic carbon. About 80% of the organic carbon in the soluble fraction was accounted for. Carbohydrates and organic acids comprised 58% of the soluble organic carbon; amino acids and anionic surface active agents comprised about 22%, of the soluble organic carbon. The predominant sugars in solution were found to be glucose and sucrose. Volatile acids identified were acetic, propionic, butyric and valeric. Acetic acid was in the greatest concentration. Twelve or more non-volatile acids were detected at low concentration. A summary of the data presented by Painter and Viney is given in Table 3.

Zuckerman (54) has characterized the soluble organic fractions of several domestic sewages by chromatographic molecular weight. This work indicates that as much as 50% of the soluble organic fraction (as measured by chemical oxygen demand) is comprised of organic compounds of about 1,200 in molecular weight.

Chemical Treatment with Lime

Lime has been widely used in the chemical treatment of wastewater for at least 100 years (33). Interest in lime treatment is increasing in view of the need for advanced waste treatment methods incorporating removal of phosphorus.

The mechanism of lime treatment traditionally has been attributed to the precipitation of carbonates from solution (36, 44). Contradictions to this theory have arisen in the past twenty-five years. Rudolfs (36) has shown that 95% of the turbidity in sewage is removed before appreciable calcium is precipitated from solution and that the addition of urine to sewage acted as a coagulant aid by reducing the lime dosage at which clarification took place. The effects of urine constituents indicated strongly that phosphate precipitation was responsible for the effects observed.

Stones (44) has shown that decarbonated samples of sewage gave the same degree of purification as the original sewage after both had been treated with lime. Coagulation by positive metallic sols was advanced as the possible mechanism of purification.

Table 3. Organic Constituents of the Soluble Fraction as determined by Painter and Viney (31)

Organic Constituent	Average Percent of Total Organic Carbon In Sewage
Carbohydrate	31.3
Volatile Acids	11.3
Non Volatile Acids	15.2
Free Amino Acids	3.1
Bound Amino Acids	7.6
Anionic Surface Active Agents	11.2
Total Organic Carbon Accounted for	79.7

Bishop (5) has reported on the results of standard jar tests on various wastewaters using a range of lime dosages. Unfiltered and filtered (0.45 u membrane filters) supernatants were analyzed for residual concentrations of total phosphorus, calcium and magnesium. The results showed that calcium concentrations increased only gradually with lime dosage up to a pH of about 10.0 while total phosphorous decreased rapidly until precipitation was essentially complete at a pH of about 10.0. This phenomenon was interpreted as the precipitation of phosphorous as hydroxyapatite ($Ca_5OH (PO_4)_3$). The phosphorous in the wastewater was being removed from solution with the calcium being added producing little net change in calcium concentration.

Mulbarger, Grossman and Dean (29) have summarized the precipitations occurring during lime clarification. These reactions in the light of Bishop's report can be summarized as follows for raw wastewater:

In the pH range up to about 10.0, lime reacts with hydrolyzed orthophosphate to form insoluble hydroxyapatite.

In the pH range from 10.0 to about 11.0 lime reacts with the carbonate alkalinity in the wastewater to form insoluble calcium carbonate.

In the pH range from about 11.0 to 11.5 insoluble magnesium hydroxide is formed.

Various authors have reported pollutant removals resulting from lime treatment in terms of several wastewater parameters including turbidity, biochemical oxygen demand, chemical oxygen demand, total organic carbon and total phosphorous.

Rudolfs (36) has shown that turbidity decreases up to a pH of about 9.5, remains constant between pH 9.5 to 10.5 and then decreases again between pH 10.5 to 11.5. There was no relation between increased clarification and the reduction of carbonates, however, phosphates caused a shift downward of the optinum pH values for clarification. Although not specifically mentioned, the increased turbidity removals reported above pH 10.5 may be due to magnesium hydroxide precipitation (5).

Owen (30) has reported 5-day biochemical oxygen demand removals in a raw wastewater of 79% after treatment with 720 mg/l of slaked lime. Buzzel and Sawyer (9) have reported that lime treatment of raw wastewater can result in 80% to 90% removal of total phosphorous and 50% to 70% of the 5-day biochemical oxygen demand.

Williamson, Heit and Calman (53) have reported chemical oxygen demand removals in a secondary effluent, filtered through Whatman No. 5 paper before treatment, of about 45%. Although a pH was not monitored, these data showed a steady reduction of a chemical oxygen demand up to a lime dosage of about 400 mg/l followed by a rapid reduction between lime dosages of 400 mg/l to 500 mg/l.

Bishop (5) has reported significant increases in clarification and total organic carbon removals in the treatment of a secondary effluent at lime dosages sufficient to produce a pH of 11.5. The increased treatment efficiency at the higher pH level was attributed to the precipitation of magnesium hydroxide which further clarified the wastewater by the removal of colloidal material.

Zuckerman (54) has reported that the high pH associated with massive lime treatment causes a breakdown of high molecular weight organics into organics of smaller molecular weight. Gel chromatography and descending paper chromatography were the primary analytical techniques used in this study.

Samples of raw sewage from various sources were analyzed for their chemical oxygen demand molecular weight distributions and it was reported that raw wastewater contains soluble organic material larger than 1,200 molecular weight and smaller soluble organics of below 400 molecular weight.

Analysis of the wastewater after treatment with lime showed evidence that the larger than 1,200 molecular weight material is altered and that only soluble organic material of low molecular weight remained. Partial removal by adsorption onto chemical floc and alkaline hydrolysis of the remainder into smaller organic molecules were advanced as the causes of the altering of the larger than 1,200 molecular weight material.

Sephadex G-15 dextran gel packed in a 2.5 cm by 45 cm chromatographic column was used extensively in the chemical treatment studies. The samples were filtered through a 0.45 membrane filter and 2 ml samples were then passed through the gel using a phosphate buffered eluent. A continuous low level chemical oxygen demand procedure was used to monitor the chromatographic eluent. The procedure used is reported to be capable of automated chemical oxygen demand analysis in the 0 to 6 mg/l range (54). Alkaline hydrolysis was further studied using descending paper chromatography and pure compounds. Organic solutions of 40 gm/l cellulose, 40 gm/l starch and 5 gm/l bovine serum albumin were subjected to pH levels of 9.0, 10.0 and 11.0 using calcium hydroxide and sodium hydroxide independently. Contact times of 50 minutes at the adjusted pH were used. The samples were then neutralized with 0.1 N sulfuric acid. Twenty-five micro-liter samples were then analyzed by descending paper chromatography. The results reported indicated that all samples adjusted to a pH of 9.0, 10.0 and 11.0 except the starch sample at 9.0, had undergone hydrolysis.

Several workers have suggested other mechanisms which may be involved in organic pollutant removals during lime treatment other than precipitation and coagulation.

Sorption has been suggested by both Rudolfs (36) and Zuckerman (54) as a possible contributing removal mechanism. A recent report by Brunner and Sproul (7) on virus inactivation during phosphate precipitation lends support to the possible significance of sorption in the removal process.

Stumm and Morgan (45) have pointed out that many naturally occurring colloidal and soluble impurities contain ionizable functional groups that are known to form complexes with polyvalent metal ions. Many such groups would carry negative charges in the pH ranges encountered during lime treatment. This suggests the possibility of chemical interactions between calcium ions and organics to promote the formation of insoluble precipitates. LaMere and Smellie (20) have demonstrated this concept by flocculating potato starch, which contains phosphoryl monoester, with cations such as Ca++.

Removals of organic pollutants, during lime treatment, are reported to be closely related to the precipitation of phosphorous and magnesium hydroxide (5, 36). Calcium carbonate precipitation appears to play a lesser role (5, 36). Removals of both soluble and colloidal impurities may be accomplished to a lesser extent through sorption and complexing (5, 20, 45, 54). Increased clarification appears to occur at lime dosages sufficient to produce a pH of about 11.5 (5, 36).

Adsorption of Soluble Organic with Activated Carbon

A review of the literature to ascertain how activated carbon adsorption takes place, what effect molecular size has and what types of organics would be considered refractory and thence appear in adsorber effluents was undertaken to aid in interpretation of carbon column effluent data.

1. Structure of Carbon and Kinetics of Adsorption

Activated carbon's adsorption properties can be attributed to its highly porous structure (24, 43). Each activated carbon particle contains a vast network of variable size pores. This great porosity provides a very large surface area for adsorbing organic molecules. A relatively small part of the surface area comprises the outside of the activated carbon particles.

Consideration of this structure indicates that the interior surface of the activated carbon must be used for effective adsorption and that adsorption of a solute molecule takes place in three consecutive steps (50). If the incidental adsorption on the exterior surface is neglected these steps can be summarized as follows:

Transport of the adsorbate to the exterior surface of the activated carbon.

Diffusion of the adsorbate into the interior surface area.

Adsorption of the adsorbate onto the interior surface area.

Observations of adsorption rate and effects of temperature, molecular size and configuration on adsorption rate indicate that the rate controlling step in rapidly mixed non-flow systems is diffusion of the adsorbate into the interior pores (28, 50). Reports on continuous flow columnar systems suggest that external transport mechanisms are rate controlling (19, 52).

2. Effect of Nature, Molecular Size and Configuration of Solute on Adsorption

Weber and Morris (28, 50, 51) have reported on the effect of molecular size and configuration of alkylbenzenesulfonates on adsorption rate and capacity. The rate of adsorption for a homologous series of ABS molecules was found to decrease with increasing molecular weight when the amount adsorbed per unit time was expressed in molar units. This effect was much less pronounced when adsorption rate was expressed in weight units.

Further experiments with alkylbenzenesulfonates (ABS) of the same molecular weight but with different alkyl chain structure indicated that those molecules having structures which permitted coiling or compactness were adsorbed at a greater rate. Investigations into the effect of molecular size on adsorption capacity indicated that high molecular weight materials are adsorbed to a greater extent than materials of lower molecular weight. As stated in a previous section, the rate limiting step in the rapidly mixed non-flow system used by Morris and Weber was indicated to be interparticle diffusion. The decrease adsorption rate with increasing molecular size can then be attributed to decreasing interparticle diffusion rates with increasing molecule size of the ABS homologs. A more compact molecule would adsorb faster in such a system for the same reason. The increase in adsorption capacity with increasing size can be attributed to the increased energy of adsorption or partition factor of higher molecular weight compounds.

Weber and Keinath (19, 52) have reported that molecular size is much less important to adsorption kinetics in fluidized adsorbers in which external transport mechanisms are rate limiting. The column adsorption systems reported on exhibited a generally higher mass transfer rate with increasing molecular size of the adsorbate.

The nature of the adsorbate must be taken into account when considering adsorptive tendency. Weber and Morris' report (28, 50) makes it clear that organic compounds of much higher molecular weight than the ABS series previously mentioned adsorb much faster even in a rapidly mixed system.

3. Organics Resistant to Activated Carbon Adsorption

There do not appear to be any conclusive reports available on the identity of the trace organics remaining in wastewater effluents after activated carbon adsorption, although attempts have been made to identify these compounds (27). Bishop et al (6) have pointed out that activated carbon does a poor job of removing colloidal organics. Strongly hydrophilic low molecular weight organics have also been suggested as an adsorption resistant group (6, 27).

Among the specific soluble organic compounds which might be expected in activated carbon adsorber effluents are sugars, amino acids, urea, low molecular weight glycols, hydroxy acids, sulfates and sulfonates (6, 27).

A review of the literature indicates that many factors can effect adsorption on organic carbon. Large colloidal organics which are limited by size in their ability to enter the activated carbon pore structure and low molecular weight hydrophilic compounds which tend to stay in the aqueous phase would be expected to be resistant to adsorption and may be present in activated carbon adsorber effluents.

The Hydrolysis-Adsorption Process

Zuckerman and Molof (54, 55) have proposed a chemical-physical treatment process which is similar to those reported by others (5, 40, 42, 49). This process would consist of the following steps (55).

Solids separation by chemical coagulation with lime plus elevation of pH to 11.5.

Reaction in a chamber to allow hydrolysis to occur.

Neutralization with acid or by recarbonation with CO_2.

Sand filtration

Activated carbon adsorption.

The reported effect of the high pH on molecules of large molecular weight (hydrolysis) was mentioned in previous sections. The hydrolysis step is the basic difference between processes which have been used previously (5, 40, 42, 49). The alleged need for such a step is based on gel chromatography studies of raw wastewater, secondary biological effluents and activated carbon adsorption effluents as well as on the interpretation by Zuckerman and Molof of data on the effect of molecular size on adsorption for a homologous series of alkylbenzenesulfonates presented by Weber and Morris (50) for a rapidly mixed non-flow adsorption system.

A summary of the major conclusions of Zuckerman and Molof (54, 55) reports are as follows:

About 25% of the organic material, passing a 0.45 micron filter, in raw wastewater is colloidal in nature.

The molecular weight distribution of the soluble organics in raw wastewater, as determined by filtering through a 0.45 micron filter and gel chromatography analysis of the filtrate, consists primarily of compounds of above 1,200 and below 400 in molecular weight.

The molecular weight distribution of the soluble organics in biological activated sludge effluents, as determined by filtering through a 0.45 micron filter and gel chromatography analysis of the filtrate, consists largely of compounds above 1,200 in molecular weight indicating that biological treatment preferentially removes low molecular weight organics.

Activated carbon adsorption processes are limited by interparticle diffusion causing the preferential removal of low molecular weight compounds.

Lime clarification removes some of the high molecular weight material in raw wastewater by adsorption onto chemical floc and the remainder are broken down by alkaline hydrolysis to more adsorbable compounds of below 400 in molecular weight.

The increased concentration of organics of below 400 in molecular weight, after the hydrolysis step, causes an increased partition factor between the liquid phase and the solid adsorbent which results in increased activated carbon adsorption capacity.

Weber (48) has commented in depth on the interpretations of previous literature on adsorption rate and capacity and on the experimental procedures reported by Zuckerman and Molof

(54, 55). These comments were based to a large extent on experimental data presented by Weber and co-workers in reports which have been cited in the preceding section (28, 50, 51). A summary of the comments presented and literature referenced are as follows:

Adsorption from solution is often enhanced by high molecular weight material (19, 50, 51, 52).

While it has been demonstrated that interparticle diffusion is rate limiting in rapidly agitated batch adsorption systems, it has also been shown that external transport mechanisms govern the column type adsorption systems generally used in wastewater treatment (19, 50, 52). If molecular weight has an effect on adsorption it would be expected to be most apparent in the batch type systems and not in the columnar type systems used in wastewater treatment.

The report by Weber and Morris (50), which included data on the effect of molecular size and adsorption for a homologous series of alkylbenzenesulphonates, clearly stated that the detrimental effect on adsorption rate with increasing molecular size was much less evident when the data was expressed in weight and not molar units.

The approximate 25% fraction of colloidal material in the filtered samples used for gel chromatography analysis could have significantly affected the gel chromatography results.

Increases in activated carbon effluent quality, which are attributed to a hydrolysis effect, could be associated with increased removal of colloidal organic materials by coagulation (5, 6).

The previously cited commentary (48) also contained the results of experiments to determine if hydrolysis of organic matter can be expected at pH 11.5 under otherwise ambient conditions and if a hydrolysis effect on activated carbon adsorption occurs. The activated carbon systems were rapidly mixed batch systems which should show the hydrolysis effect most readily. The results indicated that minor hydrolysis of proteins occurred at a pH of above 10 after several hours of contact. The results of coagulation experiments using sodium hydroxide, calcium hydroxide, and ferric chloride indicated that calcium hydroxide clarification at pH 11.5 and ferric chloride clarification at pH 6.7 produced effluents which were essentially the same in quality and that sodium hydroxide produced an effluent of much poorer quality after batch activated carbon adsorption. The organic removals attributable to the clarification process were much higher when calcium hydroxide or ferric chloride was used. The conclusion drawn from these data was that effective coagulation under less severe conditions produced the same effect on adsorption as coagulation at high pH.

There does not appear to be any comparable data on the molecular weight distribution of raw filtered wastewater, however, some data are available on the proportion of high molecular weight materials in secondary biological effluents. McDonald (25), using gel chromatography, has indicated that 70% to 80% of the soluble organic material in activated sludge effluent is of below 600 in molecular weight. Bunch, Barth and Ettinger (8), using semi-permeable membranes, have attributed about 60% of the organic material in various biological effluents to low molecular weight organics. These two reports appear to be in disagreement with data presented by Zuckerman and Molof (54, 55) indicating that activated sludge effluents contain material primarily of above 1,200 in molecular weight.

1. Pilot Plant Studies of the Hydrolysis-Adsorption Process

A pilot plant study of the hydrolysis-adsorption process has been carried out by Ecolotech Research, Incorporated under the sponsorship of the New York State Health Department (56). The pilot plant had a 10,000 gallon per day capacity and was operated for a three month period at New Rochelle, New York starting in September 1969. The process was used to treat New Rochelle raw wastewater.

The pilot plant units consisted of an upflow solids separator designed for a surface settling rate of 560 gallons per day per square foot, a hydrolysis chamber designed for a detention time of about eighty minutes, a neutralization tank utilizing sulfuric acid, a sand filter designed for a surface loading of 3.2 gallons per minute per square foot and five downflow adsorption columns, in series, totaling 20 feet of activated carbon. The activated carbon columns were loaded to produce a superficial detention time of about 60 minutes. The process sequence of unit operations was described in the preceding section.

During the period of operation, the plant achieved total average COD removals of 94% and soluble COD removals of 84%. Clarification pH was maintained above 10.5 except during plant upsets. Drops in pH were accompanied by poorer effluent quality which has been attributed by Ecolotech workers to an incomplete breakdown of high molecular weight materials. It should be pointed out again, however, that when lime clarification is used, the efficiency of the clarification process and the activated carbon column effluent is related somewhat to pH and that turbidity which is not removed by the clarification and filtration process would not be expected to be removed efficiently by the activated carbon adsorption process (5, 6) due to the inability of colloidal sized particles to enter the pore network of the activated carbon.

Chromatograms (56) obtained by direct measurement of both the New Rochelle raw wastewater and the chemically treated wastewater indicate to this writer that the majority of the organic material in the raw wastewater was of less than 1,200 molecular weight and that there was no apparent increase in concentration of the low molecular weight components. The chromatography data obtained in New Rochelle do not appear to reflect previous findings (54, 55) completely.

Gel Permeation Chromatography

Gel permeation chromatography is a method of separating or fractionating a mixture of solutes by the size of the respective solute molecules. The use of gel permeation chromatography is receiving considerable attention in the environmental science field as an analytical tool for studying the soluble organic compounds in water and wastewater. The description of these chromatographic processes has been discussed at length earlier in this report.

1. Applications of Sephadex Gels in the Water and Wastewater Field

Several workers have utilized Sephadex gels in the water and wastewater field.

McDonald (25) has used Sephadex G-10 through G-50 to fractionate the organic compounds in an activated sludge effluent. About 30% of the organic carbon in the effluent was attributed to organics with a molecular weight above 600. Hardt (15) used Sephadex G-10 and other gels to demonstrate a build-up of complex organics in a high solids biological reactor operating on a glucose substrate. Zuckerman and Molof (54, 55), as mentioned previously, have used Sephadex G-15 extensively to demonstrate changes in soluble organics after chemical-physical treatment. Smith (41) used Sephadex G-25 to fractionate concentrated kraft and sulphite process pulping wastes before and after coagulation with iron and aluminum coagulants. The predominant effect of the coagulants was the removal of high molecular weight organics from the wastes. Collins, Webb, Didwani and Lueck (10) used Sephadex G-50 to fractionate the organic components of wood pulp bleach effluents. Gjessing and Lee (14) fractionated concentrated samples of lake and stream water using various Sephadex gels. Solutes with apparent molecular weights from less than 700 to more than 200,000 were detected.

Gel permeation chromatography has been used with success to separate soluble organic compounds from complex wastewater mixtures. The molecular weights determined for such unknown mixtures of organics must be considered as approximate or apparent due to differences in molecular structure between organic groups and due to the possibility of non-steric interactions between the gel or solvent phases with some organic compounds. However, the results of gel permeation chromatography analysis should be very useful in determining the character and changes in character of soluble wastewater organics before and after treatment.

Concentration by Freeze-Drying

In investigations involving organic solutes whose chemical and physical properties are poorly defined or where analysis requires the measurement of very low concentrations, solute concentration is often desirable. In portions of this study a concentration step was deemed necessary in the preparation of samples for gel permeation chromatography in order to avoid measuring very low organics concentrated wastewater filtrates.

1. Alternative Concentration Methods, (Thin Film Evaporation and Freeze-drying)

Thin film evaporation and freeze-drying (lyophilization) were possible concentration methods which have been used in the past to facilitate the study of low concentration soluble organics in water and wastewater (17, 31, 41). These procedures and their applicabilities have been presented within Appendix A and elsewhere.

Combustion-Infrared Method for Total Carbon Content

The use of gel permeation chromatography for the separation of organic fractions requires a detector specific for organics. The detection parameter used must facilitate rapid and accurate determinations since many fractions must be analyzed in each column run. An instrument for the rapid determination of carbon would fulfill the need for such a detector.

1. Description of Method

A method for the rapid determination of total carbon has been developed within the last decade (39, 47). The method involves the rapid combustion of an aqueous sample in a stream of oxygen passing through a heated combustion tube, followed by measurement of the carbon dioxide produced. The carbon dioxide measurement is made on the gas stream with an infrared analyzer specifically sensitized for carbon dioxide. Several commercial instruments are now available from various manufacturers. A schematic of a carbonaceous analyzer is shown in Figure 1.

2. Function of the System Components

The functions of the components shown in Figure 1 are described as follows (15, 47).

Combustion of sample. The sample is injected through the sample port utilizing a microsyringe capable of measuring small volumes. A continuous pure oxygen purge passes the sample at a controlled rate into a heat resistant glass combustion tube which is maintained at a temperature of 950°C. A loosely packed plug of asbestos, impregnated with a cobalt catalyst, located at the condenser end of the combustion tube intercepts the vaporized sample. The carbon containing substances in the sample are completely oxidized to carbon

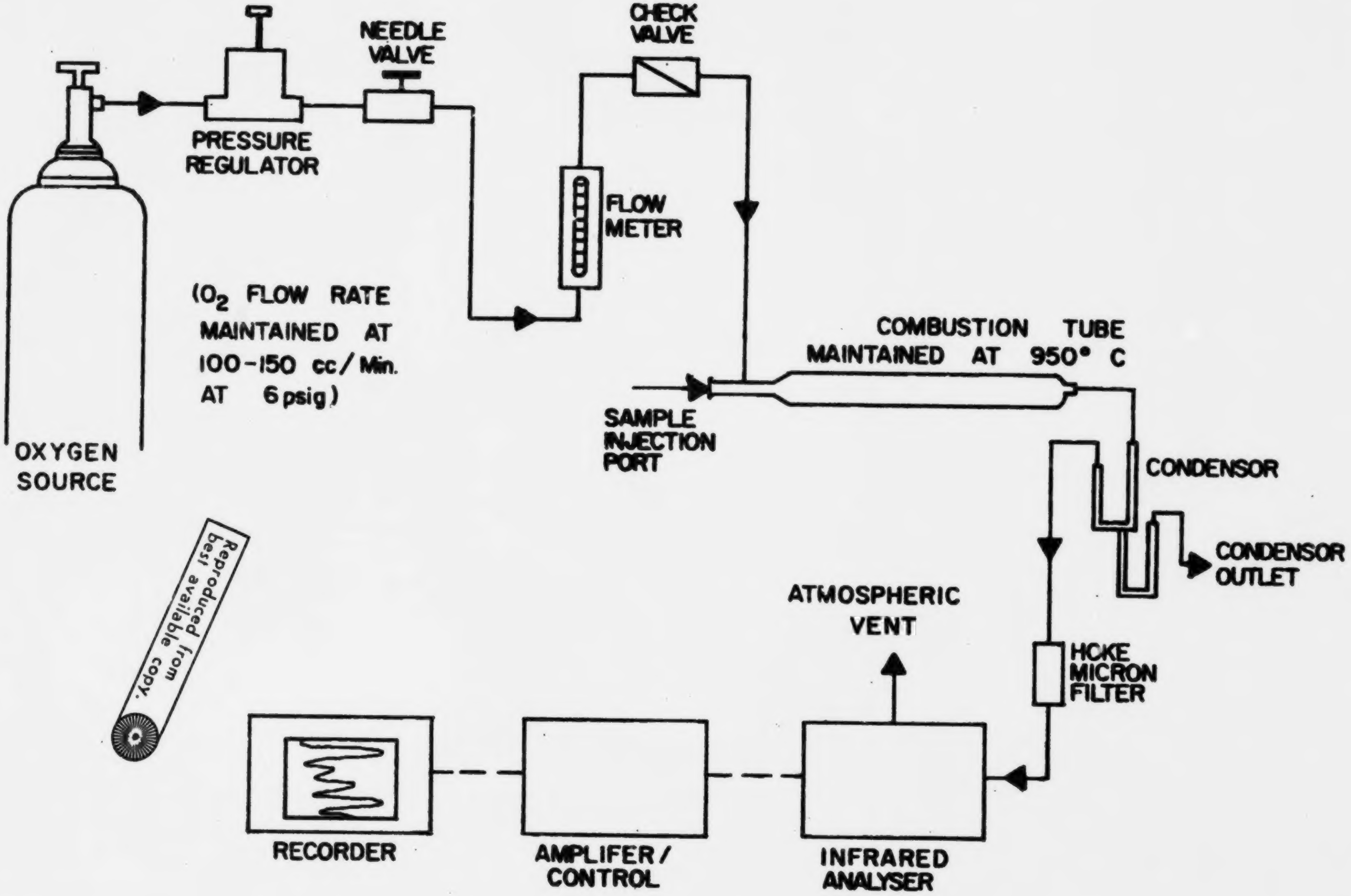
OXYGEN
SOURCE
PRESSURE
REGULATOR
NEEDLE
VALVE
FLOW
METER
CHECK
VALVE
(O_2 FLOW RATE
MAINTAINED AT
100-150 cc/Min.
AT 6 psig)
COMBUSTION TUBE
MAINTAINED AT 950° C
SAMPLE
INJECTION
PORT
CONDENSOR
CONDENSOR
OUTLET
HCKE
MICRON
FILTER
ATMOSPHERIC
VENT
RECORDER
AMPLIFER/
CONTROL
INFRARED
ANALYSER

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Fig. 1 SCHEMATIC OF INFRARED CARBONACEOUS ANALYZER

dioxide and water vapor at this point. The resulting gases pass out of the combustion tube and through a condenser where water vapor is removed.

Infrared analyzer. The carbon dioxide gas from the oxidation of the sample is passed through a filter and into the analyzer section of the instrument. The infrared analyzer section consists of two distinct components or sides. One side acts as a reference while the other side analyzes the sample stream. Each side consists of an infrared energy source which is blocked ten times per second by a two-segmented chopper, an infrared energy absorption cell and a detector cell. The adsorption cell on the reference side is filled with nitrogen gas which absorbs little of the infrared energy emitted by the reference source. The absorption cell on the sample side is of the flow through type and is placed directly in the sample train. The detector cells are filled with low pressure carbon dioxide gas and are separated by a flexible metal diaphragm.

Energy transformation. When a sample is being analyzed a portion of the infrared energy emitted by the sample source is absorbed by the carbon dioxide from the sample combustion as it passes through the sample cell. Absorption of infrared energy in the reference cell is negligible. The infrared energy transmitted out of each cell is passed into the diaphragm separated detector cells by means of infrared transmitting windows. The energy entering each detector heats the carbon dioxide gas in the detector and causes a corresponding increase in pressure. The gas in the reference detector is heated more since there is a negligible absorption of infrared energy by the nitrogen gas in the reference absorption cell. The pressure differential between the two detector cells cause the separating diaphragm to flex in relation to the energy increment between the two sides. The pressure difference varies with the amount of carbon dioxide in the sample stream and is translated electronically into a direct read out of total carbon.

3. Determination of Organic Carbon

The results from a carbonaceous analyzer include both organic carbon and any inorganic carbon in the sample unless the latter is eliminated. Van Hall et al (46, 47) found that adjustment of the sample pH to between 2 and 3, followed by purging with nitrogen gas for three to five minutes at a gas flow rate of 300 to 400 ml per minute serves to eliminate inorganic carbonates in the sample. The resulting reading reflects total organic carbon only. The nitrogen purge method was tested on various aqueous organic solutions including sugars, urea, amino acids, phenols, proteins, starches, lignins and cellulose as well as on various wastewaters (39, 47). No loss of organic acids, methanol, or phenol occurs but compounds that are both volatile and slightly soluble in water, such as light hydrocarbons, are swept out of the sample. Hardt (15) has confirmed the general method of Van Hall et al (47) using prepared solutions of sodium carbonate. A nitrogen flow rate of 500 ml per minute for five minutes, after acidification of the samples, was used during the purging procedure.

4. Applicability of the Carbonaceous Analyzer to Wastewater Analysis

Schaffer et al (39) have reported on the application of the carbon analyzer in wastewater analysis. The results of analyses by the infrared method were compared with carbon analyses by a wet-oxidation method. The suitability, accuracy, and precision of the infrared method were verified.

Three recent studies, utilizing gel permeation chromatography for soluble wastewater organics, have also indicated the applicability of organic carbon by the infrared method, as a detection parameter (15, 25, 41).

Organic carbon determinations by the combustion infrared method have been shown to be accurate and applicable to the analysis of wastewater organics. The usefulness of this method for analyzing gel permeation chromatography eluents has been recognized by several workers (13, 15, 25, 41).

SECTION IV: PROCEDURES

Operational Procedures

1. Sample Preservation

Preliminary studies indicated that a reduction in organic material occurred unless wastewater samples were preserved. Refrigeration at 4°C or chemical preservation with mercuric chloride have been used in past studies (17, 44) and were found to be acceptable for this study.

All waste samples were routinely kept in the 4°C cold room after arrival in the laboratory and all analyses were completed as soon as possible. In addition, all samples collected with the exception of the Eastern-Western sewage sample of June 30, 1969, were preserved, at the time of collection, by adding 1 ml of a 40 g/l mercuric chloride solution to each liter of sample. The aforementioned exception was packed in ice immediately in lieu of chemical preservation.

All samples obtained from the New York State Health Department, Research Unit, had been kept under refrigeration continuously after collection with the exception of the New Rochelle samples of October 19, 1969 which underwent an approximate 5 hour transit time from New Rochelle to Albany, New York, without preservation. These samples were refrigerated upon arrival in Albany.

2. Definition of the Soluble Organic Fraction

The soluble organic fraction is defined in this study as that fraction of wastewater organics which pass a 0.45 micron pore size membrane filter (54, 55). Organic material, in all cases, was measured as organic carbon. The organic carbon content of a sample after membrane filtration is termed soluble organic carbon in the text from this point on.

3. Jar Tests

One and one-half liter aliquots of selected waste samples were chemically treated in the laboratory with either sodium hydroxide (NaOH) or calcium hydroxide ($Ca(OH)_2$). The sequential procedure used in the jar tests was as follows:

Determination of initial pH and initial soluble organic carbon.

Flash mix at 100 rpm while chemicals were added by pipette, from stock solutions, over a one minute period.

Equilibration for one minute and determination of pH.

Reduction of paddle speed to 20 rpm and flocculation for a 20 minute period.

Stop paddle and settle for a 30 minute period.

Membrane filter supernatant, neutralize filtrate with sulphuric acid (H_2SO_4) to a pH between 7.0 and 7.6 and determine soluble organic carbon content of neutralized filtrate.

Chemical dosages were computed from the volume and concentration of chemical added to the 1.5 liter aliquot of waste.

4. Sample Concentration by Freeze-Drying

All samples to be concentrated were prepared by membrane filtering (0.45 micron) and neutralization to a pH of 7.0 and 7.6 (if this had not been done previously). The sequential procedure used was as follows:

Shell freeze a 500 ml aliquot of the sample in an acetone and dry ice mixture.

Fill freeze-dryer condenser with acetone and dry ice and evacuate the system to a vacuum of 20 microns of mercury.

Place sample bottle on freeze-dryer port and open three way valve to vacuum.

Freeze-dry sample for about 24 hours.

Remove sample bottle and add additional 250 ml to 400 ml aliquots of sample as necessary.

Refreeze and concentrate further.

Melt finished sample and membrane filter (0.45 micron) liquid concentrates.

Place filtered concentrates in tightly capped test tubes and store at 4°C for future use.

Subsequent to the completion of the above procedure, recovery of soluble organic carbon was calculated. Organic carbon concentration in the filtered concentrate was measured and combined with the volume of the concentrate to provide the total amount of organic carbon in such concentrate. These data, combined with the amount of organic carbon in the original sample, allowed for the calculation of the recovery factor.

Analytical Procedures

1. Organic Carbon Determination

Organic carbon determinations were made using either the Beckman or the modified carbonaceous analyzer both of which have been described previously.

The Beckman instrument was calibrated with 20 micro-liter samples to produce a full scale deflection of the recorder at 100 mg/l of organic carbon.

The modified instrument was calibrated with 50 micro-liter samples of oxalic acid solutions to produce a full scale deflection of 10 mg/l of organic carbon on low range and with 40 micro-liter samples to produce a full scale deflection of 100 mg/l of organic carbon on high range.

A Hamilton Model SK 148, 50 micro-liter graduated syringe was used to inject all samples.

The calibrations of the Beckman instrument and the low range of the modified instrument were linear. The calibration of the modified instrument on high range was not quite linear and organic carbon was determined from a calibration curve of standard organic carbon versus recorder reading.

Inorganic carbon was eliminated by acidification of a 5 ml aliquot of sample of pH 2.0 with 2N hydrochloric acid (HCl) and purging for five minutes with nitrogen gas at 500 cc/min. This procedure follows that recommended in the literature (15, 46) and is termed the conventional method from this point on. In instances where a sample contained more than

100 mg/l of organic carbon an appropriate dilution was made with double distilled water.

2. Gel Permeation chromatography

Organic solutes in the soluble organic fraction of wastewater concentrates were separated (fractionated) by molecular size using gel permeation chromatography (see Figures 2 and 3).

The procedure used was as follows:

Preparation of Gel columns. The Sephadex G-15 gel used was completely swollen in an excess of phosphate buffered eluent for a minimum of 48 hours. The column was then packed to a gel bed height of 91 cm for both upflow and downflow modifications. The procedure used to pack the chromatographi column was as follows:

The column, with an upflow adaptor installed in the bottom, was mounted vertically in the cold room on a metal frame.

A funnel fitted with a rubber stopper was placed in the top of the column and the column was completely filled with phosphate buffered eluent.

The swollen gel suspension was placed in the funnel in one pour and stirred constantly with a glass rod as the gel particles settled to the bottom of the column.

The lower outlet of the column was opened to permit eluent flow after a few centimeters of the gel bed has built up in the bottom of the column.

When the gel bed was completely formed to a height of 91 cm, it was washed with several bed volumes of eluent, using about the same flow rate that would be used for sample elution (40 ml/hr).

The column was then capped for downflow or upflow operation by either placing a nylon sample net over the top surface of the gel and screwing on a plastic cap connected to the eluent reservoir (downflow) or by connecting an upflow adaptor to the eluent reservoir, installing it in the top of the column and inverting the column so that the eluent entered

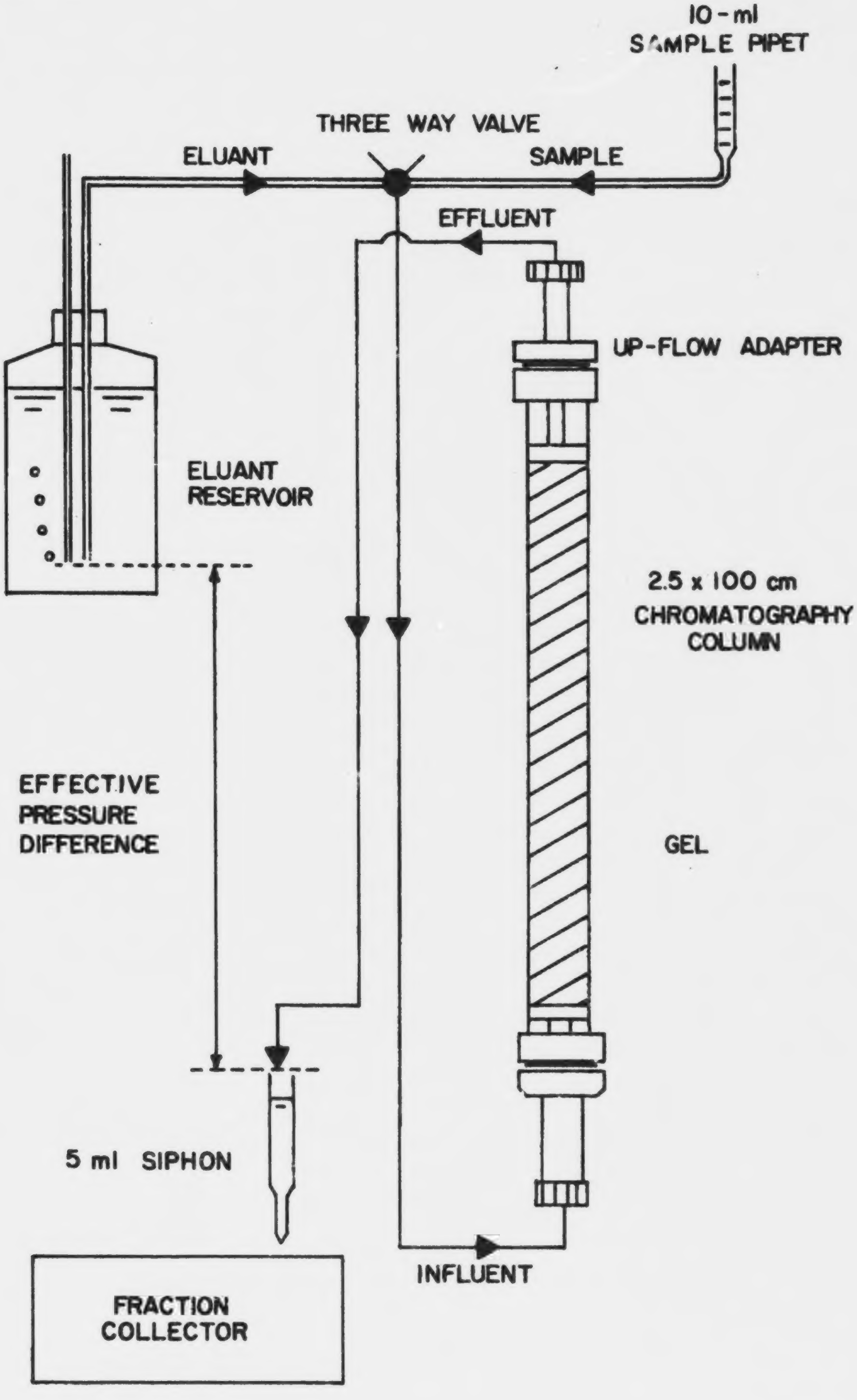
10-ml
SAMPLE PIPET
THREE WAY VALVE
ELUANT
SAMPLE
EFFLUENT
UP-FLOW ADAPTER
ELUANT
RESERVOIR
2.5 x 100 cm
CHROMATOGRAPHY
COLUMN
EFFECTIVE
PRESSURE
DIFFERENCE
GEL
5 ml SIPHON
INFLUENT
FRACTION
COLLECTOR

Fig. 2 SCHEMATIC OF UPFLOW GEL COLUMN

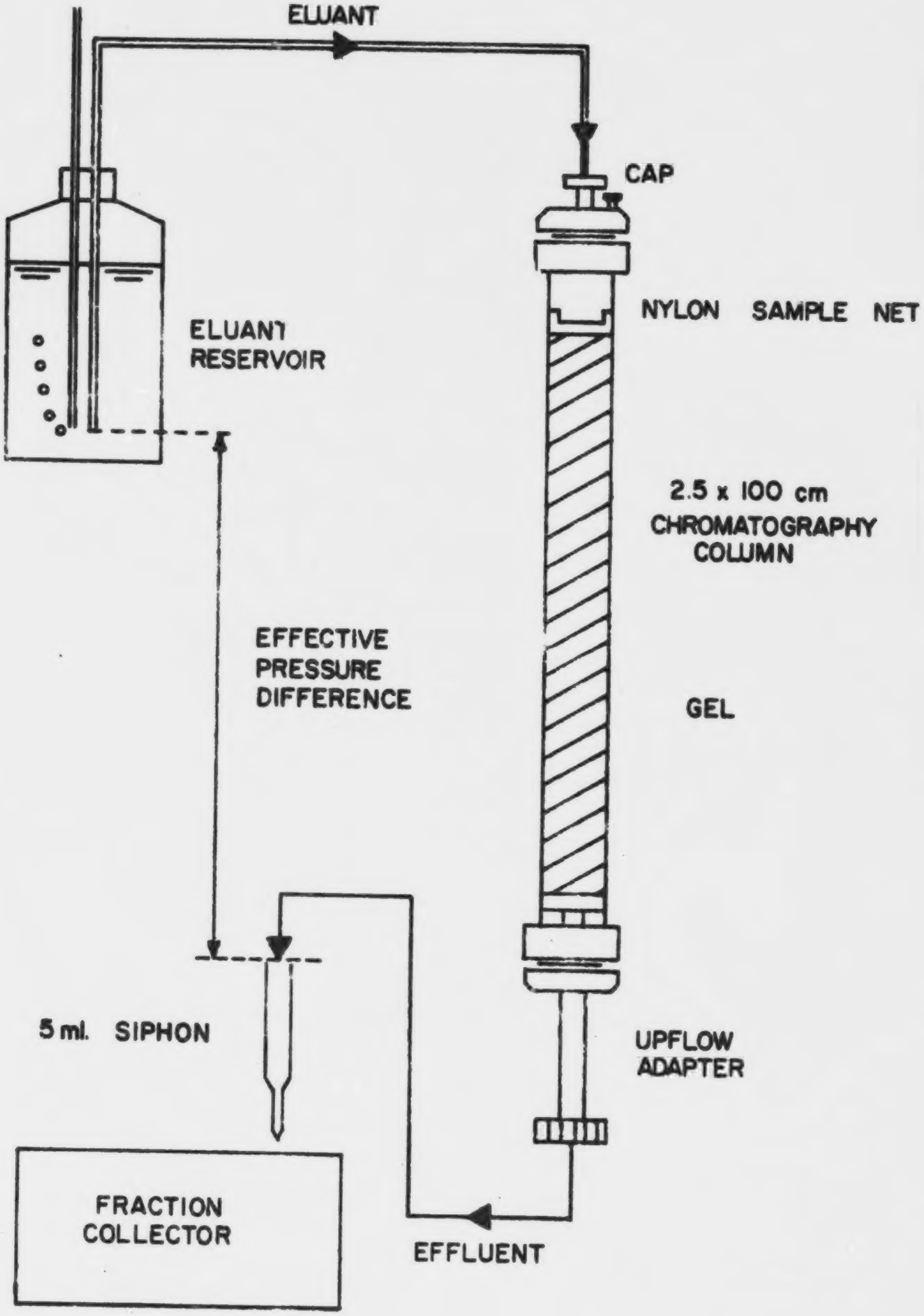
ELUANT
CAP
NYLON SAMPLE NET
ELUANT RESERVOIR
2.5 x 100 cm
CHROMATOGRAPHY COLUMN
EFFECTIVE PRESSURE DIFFERENCE
GEL
5 ml. SIPHON
UPFLOW ADAPTER
FRACTION COLLECTOR
EFFLUENT

Fig. 3 Schematic of Downflow Gel Column

the column from the bottom (upflow).

The elevation of the eluent reservoir was adjusted to produce a flow rate into the fraction collector of 40 ml/hr.

Standardization. The packed columns were standardized for apparent molecular weight (a.m.w.) determinations by eluting 2 ml to 3 ml volumes of the standards and collecting each fractionated standard in 5 ml aliquots with the fraction collector.

Each fraction was analyzed for organic carbon or conductivity in the case of standard No. 3. The fractions at which the solutes in the standards were eluted in greatest concentration were taken as their elution fraction (11). The logarithm of the molecular weight of the respective solute was then plotted against its elution fraction to develop a standard curve for apparent molecular weight determinations.

Sample application. Samples were applied either directly to the top of the gel bed by pipette in the case of the downflow column or automatically from a 10 ml sample pipette in the case of the upflow column. The direct application procedure required removal of all the eluent from the top of the gel bed, applying and drawing the sample into the gel, two successive, 5 ml applications of eluent and then refilling the top of the column and continuously eluting the sample via the eluent in the reservoir.

Fractionation and examination of waste samples. Samples of filtered wastewaters and wastewater concentrates were placed on the columns in volumes ranging from about 1% to 2% of the gel bed volume (5 ml to 10 ml). Fractions equalling about 115% of the gel bed volume (105-five ml fractions) were then collected and organic carbon determinations were made on each fraction. The conventional organic carbon determination procedure was used. No organic carbon appeared in any of the fractions before fraction 35 in any of the samples analyzed.

Chromatograms were obtained for each sample fractionated by plotting fraction number versus organic carbon and connecting the plotted points with a smooth curve. The appearance of definite peaks in the chromatogram signified the presence of organic solutes, of approximately the same apparent molecular size, in relatively high total concentration. The presence of a peak when fractionating a mixture of unknown organic solutes does not necessarily signify the presence of a single compound as was the case with standard elutions. A lack of definite peaks signified the presence of organic solutes over a range of molecular sizes in insufficient concentration to produce distinct peaks.

The apparent molecular weight of a peak was determined from the standard curve for the column and represents the approximate average apparent molecular weight of the organic contained in the peak. Molecular weight determinations are termed apparent because unknown organic mixtures were being analyzed and because of the references to non-steric behavior cited previously (11, 23).

When it was desired to compare samples treated to different degrees, the concentrates being compared were either diluted to contain the same organic carbon content or the column sample volumes were adjusted to produce the same result. Chromatograms obtained in this manner could be compared directly to determine relative changes in peaks.

Calculation of percentages of organic carbon in peaks was made by computing the percentage of organic carbon recovered in the respective peak according to the following relationship

$$\frac{C_o \times N_o}{C_1 \times N_1} \times 100 = \%$$

where:

C_o = Average Organic Carbon in Peak's Fractions (mg/l)
C_1 = Average Organic Carbon in all Fractions (mg/l)
N_o = Number of Fractions in Peak
N_1 = Number of Fractions Analyzed

The percentage obtained was then multiplied by the soluble organic carbon in the sample before concentration to obtain the equivalent amount of soluble organic carbon in the original sample. It will be shown that no significant changes in the organics occurred during the concentration process making the results of this calculation valid.

The recovery of organic carbon from the gel column was calculated according to the following relation:

$$\frac{\text{Total Vol Eluted (ml)} \times \text{Average Organic Carbon (mg/l)}}{\text{Sample Vol (ml)} \times \text{Sample Organic Carbon (mg/l)}} \times 100 = \%$$

Experimental Procedures

Actual methodologies were conducted for this aspect of these investigations in four distinct experimental phases. These phases are described below.

The preparation and standardization of the G-15 upflow gel column was done according to McDonald (25). Moreover, a considerable amount of preliminary experimentation with various buffer systems and Sephadex gels, with various fractionation ranges, occurred in order to better understand the intricacies involved in this particular phase of the study.

Laboratory chemical treatment studies were done to obtain data on the molecular size distribution of the soluble organics in untreated wastewater and to determine if chemical treatment with calcium hydroxide caused significant changes in molecular size distributions.

The upflow column, freeze-dryer, Beckman carbonaceous analyzer and jar test procedure were used throughout. The sequence of operational and analytical procedures followed is shown in Figure 4.

The primary objective of the treatment studies via chemical-physical means was to obtain data on the molecular size distributions of chemical-physical effluents. The upflow column, freeze-dryer and Beckman carbonaceous analyzer were again used. All samples analyzed were supplied by the New York State Health Department, Research Unit. The sequence of operation and analytical procedures used is shown in Figure 5.

Analysis of Unconcentrated Samples and Validation of Freeze-Drying

The analysis of unconcentrated samples and validation of freeze-drying done to obtain further data on the effects of pH on molecular size distribution in sewage and to validate the freeze-drying procedure. Both freeze-dryed and unconcentrated samples were analyzed. The gel column was modified

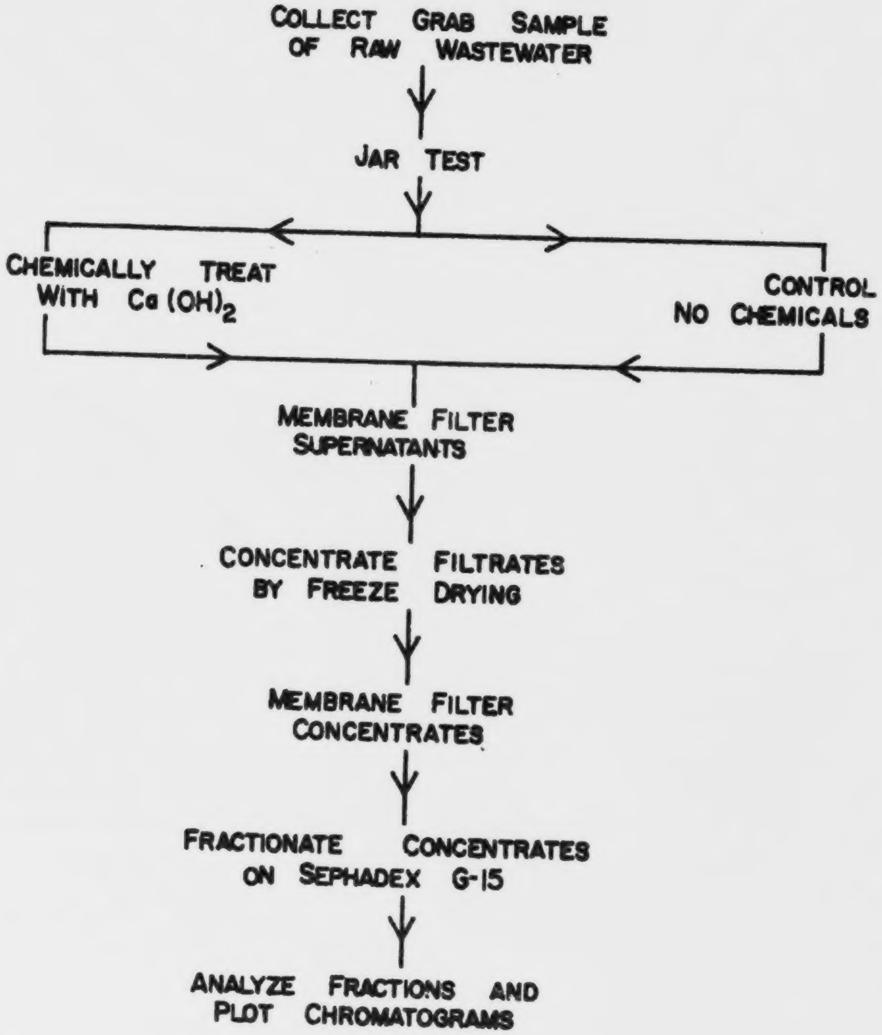
COLLECT GRAB SAMPLE OF RAW WASTEWATER
JAR TEST
CHEMICALLY TREAT WITH Ca (OH)2
CONTROL NO CHEMICALS
MEMBRANE FILTER SUPERNATANTS
CONCENTRATE FILTRATES BY FREEZE DRYING
MEMBRANE FILTER CONCENTRATES
FRACTIONATE CONCENTRATES ON SEPHADEX G-15
ANALYZE FRACTIONS AND PLOT CHROMATOGRAMS

Fig. 4 EXPERIMENTAL PROCEDURE USED DURING LABORATORY CHEMICAL TREATMENT STUDIES.

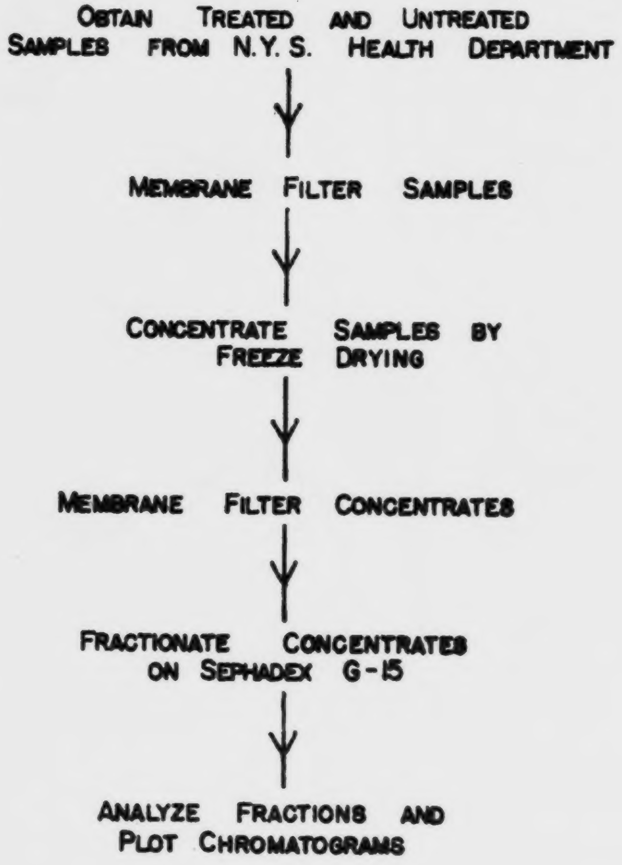
OBTAIN TREATED AND UNTREATED SAMPLES FROM N.Y.S. HEALTH DEPARTMENT
MEMBRANE FILTER SAMPLES
CONCENTRATE SAMPLES BY FREEZE DRYING
MEMBRANE FILTER CONCENTRATES
FRACTIONATE CONCENTRATES ON SEPHADEX G-15
ANALYZE FRACTIONS AND PLOT CHROMATOGRAMS

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Fig. 5 EXPERIMENTAL PROCEDURE USED DURING CHEMICAL - PHYSICAL TREATMENT STUDIES

for downflow operation at the start of this phase and the modified carbonaceous analyzer (high and low range as applicable) was used to analyze fractions. Only the Elnora sewage sample was analyzed. The sequence of operational and analytical procedures followed is shown in Figure 6.

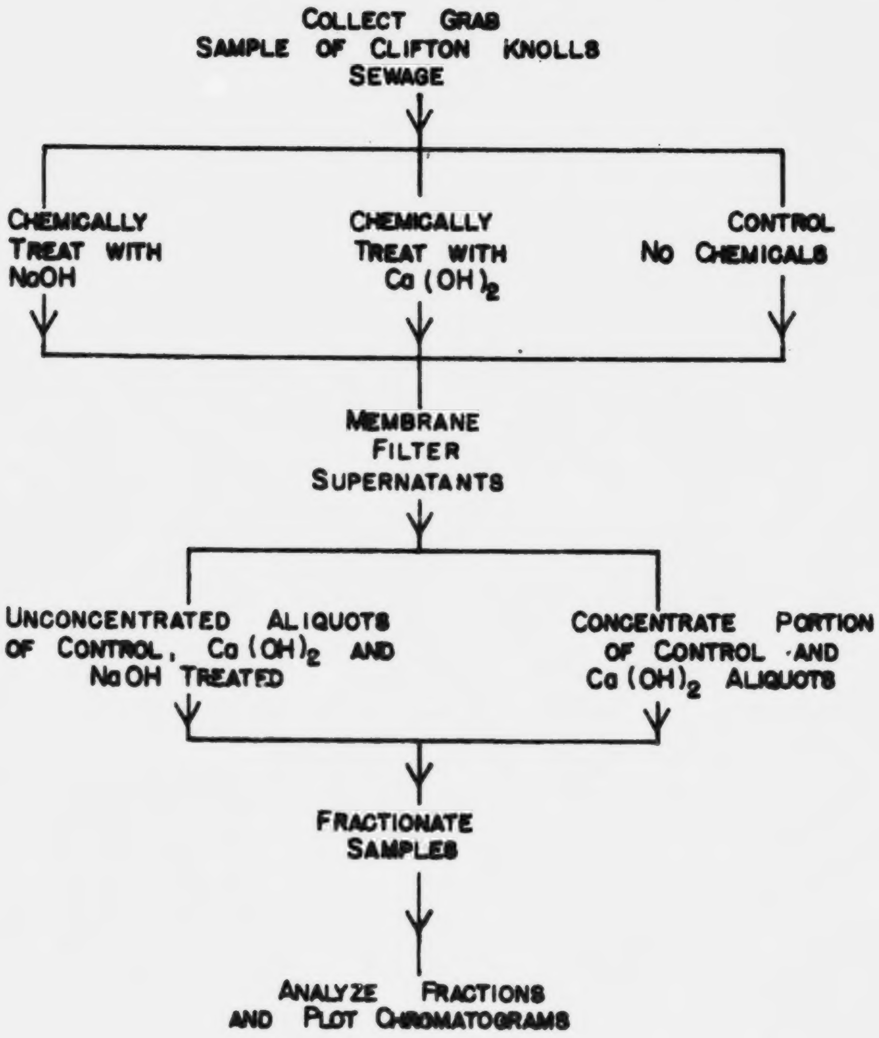
COLLECT GRAB SAMPLE OF CLIFTON KNOLLS SEWAGE
CHEMICALLY TREAT WITH NaOH
CHEMICALLY TREAT WITH Ca(OH)2
CONTROL NO CHEMICALS
MEMBRANE FILTER SUPERNATANTS
UNCONCENTRATED ALIQUOTS OF CONTROL, Ca(OH)2 AND NaOH TREATED
CONCENTRATE PORTION OF CONTROL AND Ca(OH)2 ALIQUOTS
FRACTIONATE SAMPLES
ANALYZE FRACTIONS AND PLOT CHROMATOGRAMS

Fig. 6 EXPERIMENTAL PROCEDURES USED DURING ANALYSIS OF UNCONCENTRATED SAMPLES AND VALIDATION OF FREEZE DRYING.

SECTION V: RESULTS AND DATA ANALYSIS

1. Gel Column Standardization

Several runs were made with standards for both the upflow and downflow column modifications. The results were identical for both types. The elution characteristics of each standard are tabulated in Table 4. The fraction in which the standard appeared in maximum concentration was taken as its elution fraction (11).

The standard curve developed from these data is shown in Figure 7. Inspection of this figure reveals that the standards were eluted in the order of decreasing molecular weight and that the relationship of elution fraction versus the logarithm of molecular weight was approximately linear. Figure 7 was subsequently used to determine the apparent molecular weights of the unknown organic compounds in the samples analyzed by chromatography.

The calculated bed volumes of both column modifications were 446 ml or 89-5 ml fractions; therefore, organics eluted fraction 89 are under non-steric influences ($Kav > 1$) and cannot be defined as to apparent molecular weight.

2. Laboratory Chemical Treatment Studies

Introduction. The laboratory chemical treatment studies were conducted using the procedure shown in Figure 4. The Eastern-Western sewage of June 6, 1969 and the Waterford sewage of June 24, 1969 were analyzed before and after lime treatment while the Eastern-Western sewage of June 30, 1969 was analyzed untreated only. A summary of chemical treatment, organic carbon and organic carbon recovery data is presented in Table 5. Chemical treatment was carried well beyond pH 11 and a significant decrease in soluble organic carbon occurred in all sewage samples treated with lime. Freeze-dryer recoveries of soluble organic carbon averaged about 75% while recoveries of soluble organic carbon from the column averaged about 94% giving an overall recovery of about 71%.

Chromatographic Results - Eastern-Western Sewage. Chromatograms for the concentrates of the Eastern-Western sewage of June 6, 1969 are shown in Figure 8. The untreated concentrate

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Table 4. Elution Characteristics of Standards Fractionated on 2.5 cm x 91 cm G-15 Sephadex Gel Columns

Standard	Molecular Weight	Elution Fraction
Sodium Chloride	59	82
Glucose	180	68
Sucrose	342	62
Raffinose	594	54
Egg Albumin	45,000	39
Blue Dextran	About 2,000,000	39

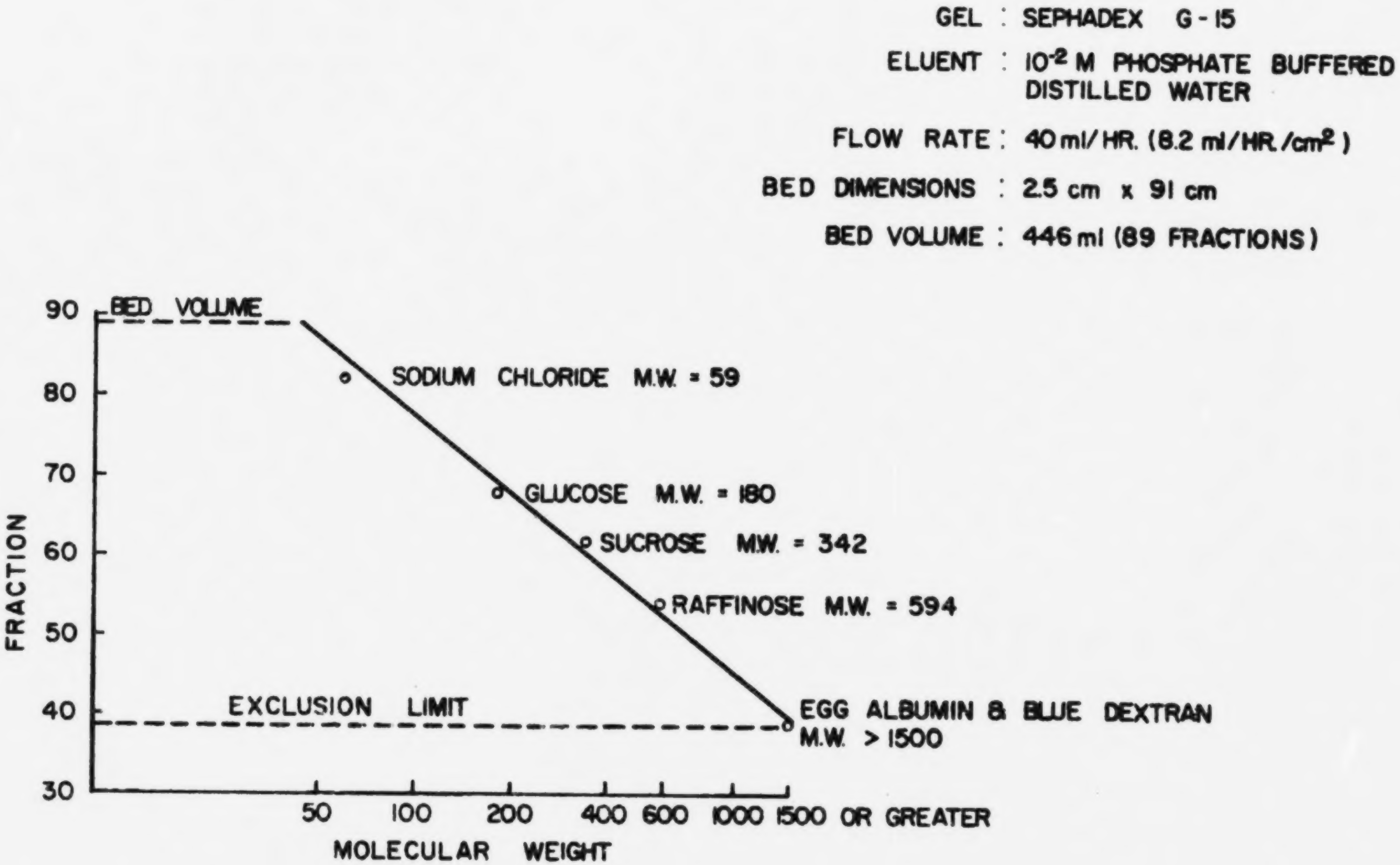

Fig. 7 STANDARD CURVE FOR APPARENT MOLECULAR WEIGHT DETERMINATIONS.

Table 5. Treatment and Carbon Recovery Data Laboratory Chemical Treatment Studies

Sewage Sample	$Ca(OH)_2$ Dosage mg/l	pH Attained	Soluble Organic Carbon mg/l		Dryer Recovery % Org. C	Column Recovery % Org. C
			Before Treatment	After Treatment		
Eastern Western 6/6/69						
Aliquot 1	None	----	75	75	78	95
Aliquot 2	600	11.6	75	65	72	90
Eastern Western 6/24/69	None	----	56	56	85	107
Waterford 6/24/69						
Aliquot 1	None	----	76	76	80%	92
Aliquot 2	107	9.6	75	63	68%	94
Aliquot 3	267	11.2	76	55	74%	93

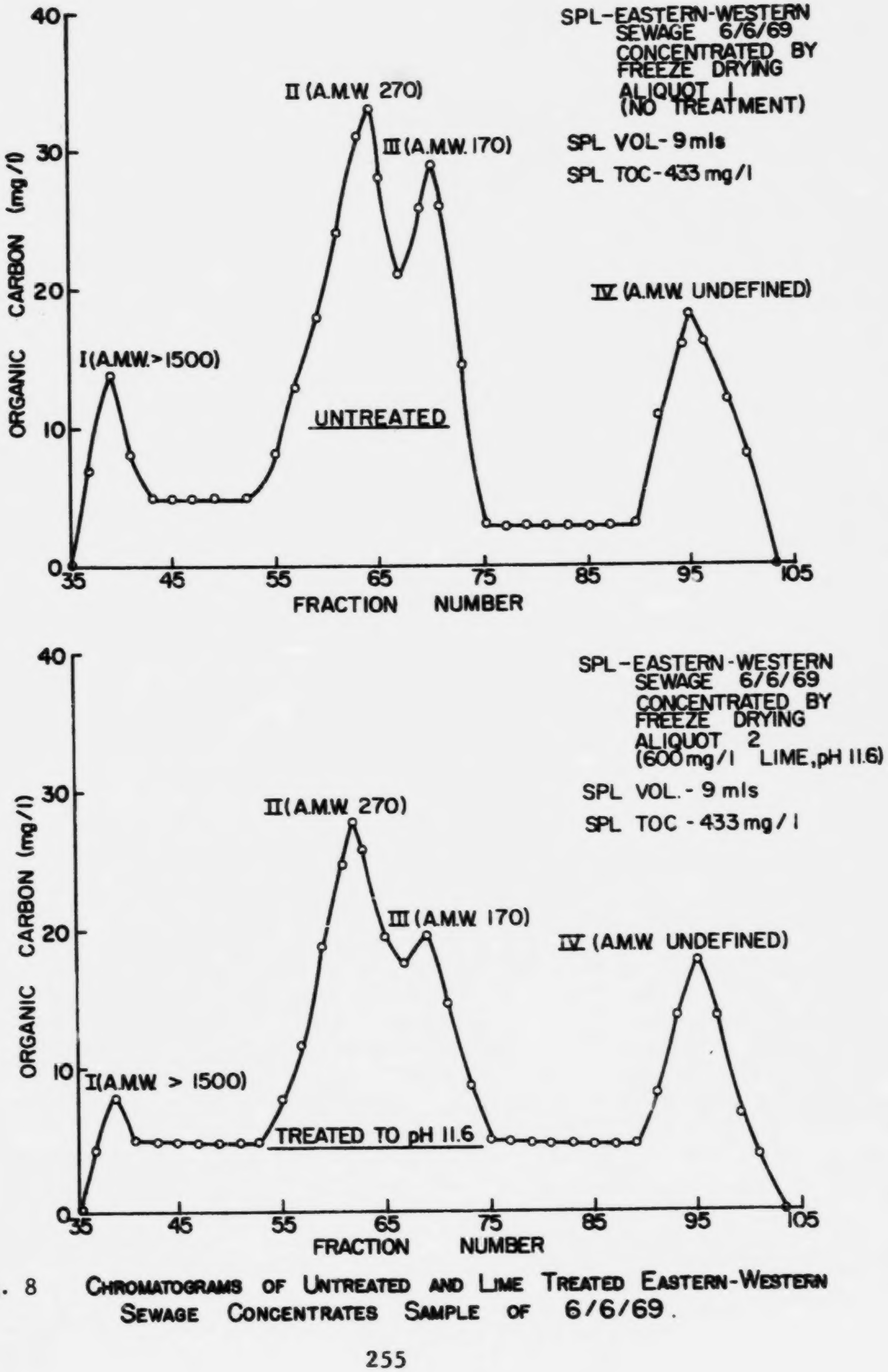

Fig. 8 Chromatograms of Untreated and Lime Treated Eastern-Western Sewage Concentrates Sample of 6/6/69.

produced four distinct peaks with apparent molecular weights as noted in the chromatogram. The great majority of the soluble organic carbon was eluted well below the exclusion limit of the gel (apparent molecular weight 1,500). Peak IV was eluted after the total bed volume (Kav > 1) and cannot be defined as to apparent molecular weight. The concentrate derived from the lime treated sewage produced a similar chromatogram which exhibits the same peaks, however, all but peak IV showed a decrease in magnitude.

Table 6 summarizes the percentages of recovered soluble organic carbon in each peak and the calculated soluble organic carbon concentration of each peak in the organic unconcentrated sample. This table indicates that all peaks with the exception of peak IV decreased with chemical treatment. Only 8.7% of the soluble organic carbon in the untreated sewage concentrate was within the exclusion peak (≥ 1,500 apparent molecular weight) indicating a very low percentage of high molecular weight soluble organics in the sewage.

Figure 9 and Table 7 present the data obtained from analysis of the Eastern-Western sewage of June 30, 1969. The sewage concentrate produced a chromatogram strikingly similar to the previous sample. The same four peaks were present although the relative magnitudes of the peaks were somewhat different; only 3.6% of the soluble organic carbon in the concentrate was within the exclusion peak indicating again a very low percentage of high molecular weight soluble organics in the sewage.

Chromatographic results - Waterford sewage. Chromatograms for the three concentrates of Waterford sewage are shown in Figure 10. The untreated concentrate produced a chromatogram which was again strikingly similar to both of the previous chromatograms for untreated Eastern-Western sewage. The chromatograms of the concentrates obtained after treatment with 107 mg/l of lime and 267 mg/l of lime indicate the presence of all original peaks and little relative change. Peak II increased slightly in magnitude and peak III decreased significantly. Peaks I and IV changed little.

Table 8 summarizes the percentages of recovered soluble organic carbon in each peak and the calculated soluble organic carbon concentration of each peak in the original unconcentrated sample. This table shows that only 6.1% of the soluble

Table 6. Effect of Chemical Treatment on Eastern-Western Sewage of 6/6/69

Peak	Apparent Molecular Weight	% Soluble Organic Carbon in Peaks		Calculated Organic Carbon In Original Sample mg/1	
		Aliquot 1	Aliquot 2	Aliquot 1	Aliquot 2
I	1,500	8.7	5.3	6.5	3.4
II	270	38.6	36.4	29.0	23.6
III	170	23.6	17.2	17.7	11.2
IV	Undefined Kav > 1	18.3	18.5	13.7	12.0

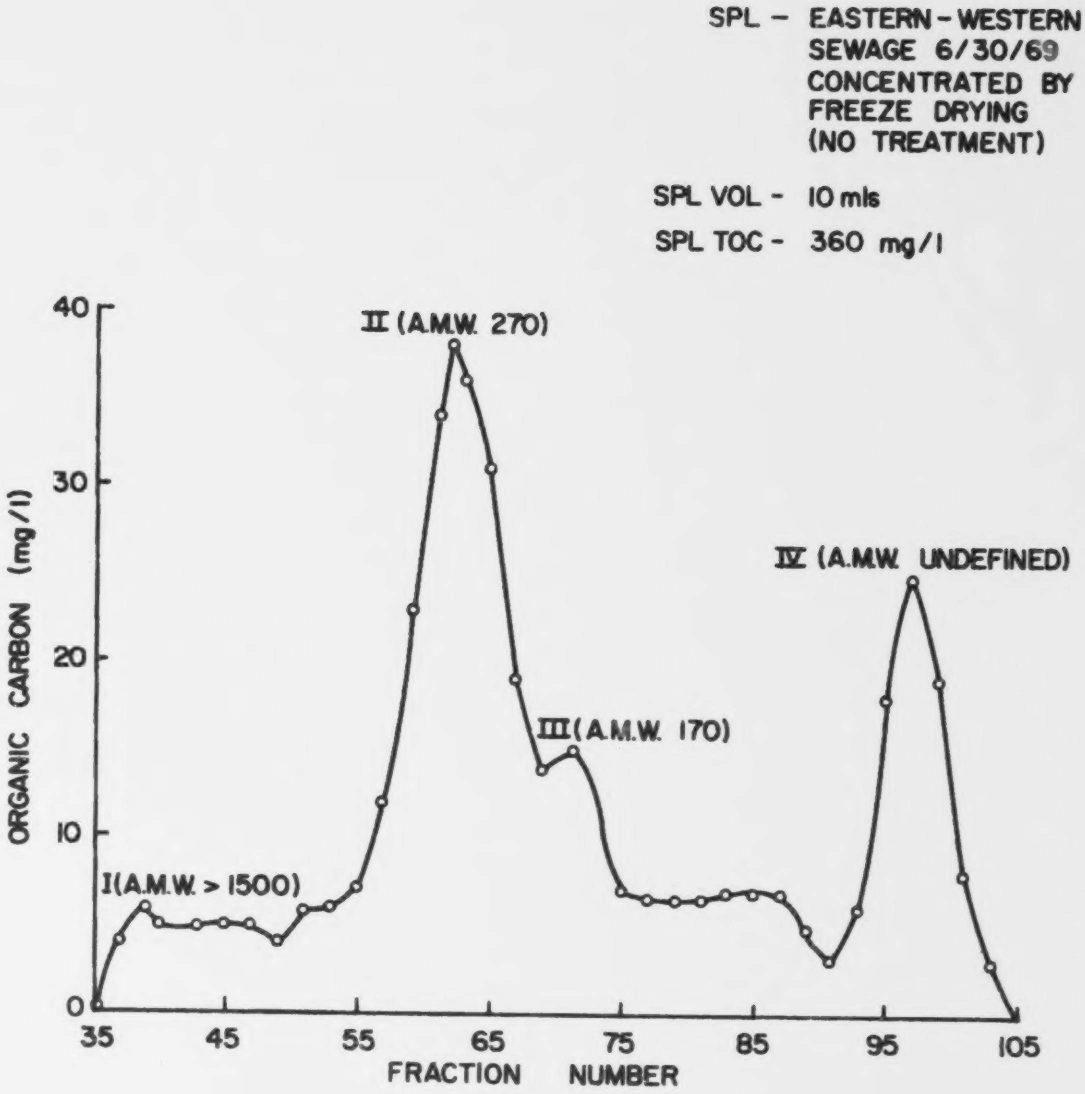

Fig. 9 CHROMATOGRAM OF EASTERN-WESTERN SEWAGE SAMPLE OF 6/30/69.

Table 7. Apparent Molecular Weight Distribution of Eastern-Western Sewage of 6/30/69

Peak	Apparent Molecular Weight	% Recovered Organic Carbon in Peak	Calculated Organic Carbon in Original Sample (mg/1)
I	1,500	3.6	2.0
II	300	45.1	25.2
III	220	9.2	5.2
IV	Undefined Kav > 1	20.1	11.3

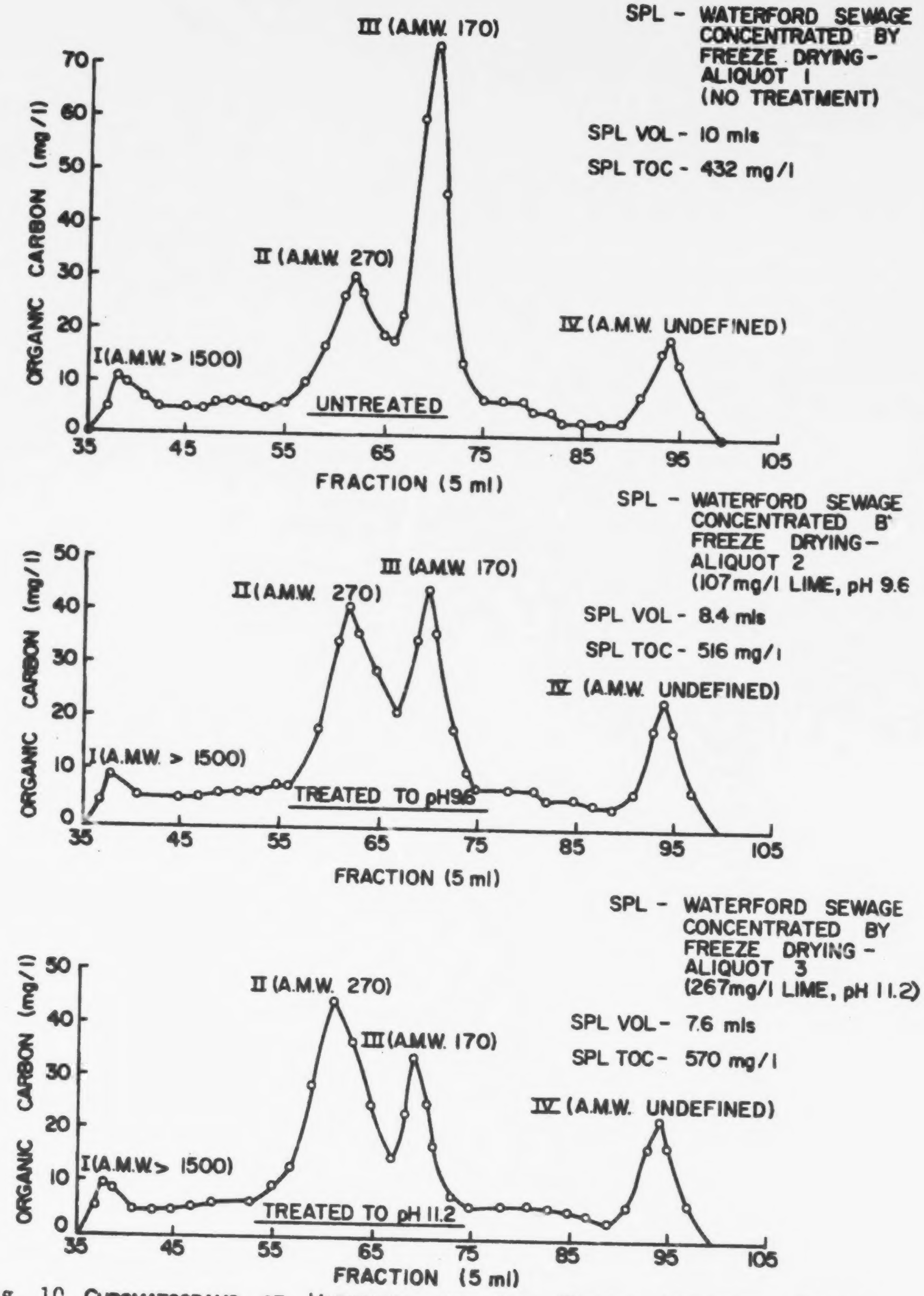

Fig. 10 CHROMATOGRAMS OF UNTREATED AND LIME TREATED WATERFORD SEWAGE.

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Table 8. Effect of Chemical Treatment on Waterford Sewage

Peak	Apparent Molecular Weight	% Recovered Soluble Organic Carbon in Peak			Calculated Soluble Organic Carbon in Original Sample (mg/1)		
		Aliquot 1	Aliquot 2	Aliquot 3	Aliquot 1	Aliquot 2	Aliquot 3
I	1,500	6.1	4.7	5.0	4.6	3.0	2.8
II	300	29.8	37.2	43.7	21.8	23.5	24.0
III	170	39.6	26.8	19.3	30.1	16.7	10.6
IV	Undefined Kav> 1	11.9	12.9	13.2	9.1	8.1	7.3

Aliquot 1 = No chemical treatment

Aliquot 2 = 107 mg/1 $Ca(OH)_2$ to pH 9.6

Aliquot 3 = 267 mg/1 $Ca(OH)_2$ to pH 11.2

organic carbon in the untreated sewage concentrate was within the exclusion peak indicating a very low percentage of high molecular weight soluble organic in the sewage. Peaks I and III decreased with chemical treatment; however, a lime dosage of 267 mg/l did not substantially change the effect brought about with a 107 mg/l lime dosage.

3. Chemical Physical Treatment Studies

Introduction. The New York State pilot plant samples analyzed during this phase were composites which allowed direct comparison of aliquots taken at different points on the treatment process. The New Rochelle sample set was not collected in relation to pilot plant detention times and therefore no attempt is made to compare between the various aliquots.

The chemical treatment and soluble organic carbon recovery data for each sample set are presented in Table 9. No lime dosage or pH data are available for the New Rochelle sample set. The chemical treatment effluent has been neutralized at the pilot plant site. The plant was normally operated using a pH in excess of 10.5 (56). Analysis was made using the procedure shown in Figure 4.

New York State pilot plant samples.

Sample set of 9/26/69. Chromatograms of the concentrated influent and carbon column effluent for 9/26/69 are shown in Figure 11. Both chromatograms show five peaks and both chromatograms closely resemble each other. The carbon column effluent concentrate contained predominently low apparent molecular weight material.

Table 10 presents data on percentages of soluble organic carbon and soluble organic carbon concentrations in each aliquot. These data are expressed in terms of apparent molecular weight ranges for ease of presentation. Percentage removals for each range are also presented. About 80% of the concentrated influent soluble organic carbon was under 1,000 in apparent molecular weight. The carbon column effluent concentrate contained about 70% of its soluble organic carbon below 1,000 in apparent molecular weight. The percentage removals in all ranges were quite good, however, the above 1,000 apparent molecular weight material was removed

Table 9. Treatment and Soluble Organic Carbon Recovery Data Chemical Physical Treatment Studies

Sample	Location In Treatment Process	Chemical Dosages Used	pH Attained	Soluble Organic Carbon	Freeze Dryer Recovery %	Column Recovery %
NYS Pilot Plant 9/26/69		88.5 mg/l $Ca(OH)_2$ and 17.7 mg/l $FeCl_3$	9.5			
Aliquot 1	Untreated			40	60	95
Aliquot 2	Carbon Column Effluent			7	85	102
NYS Pilot Plant 10/10/69						
Aliquot 1	Untreated	222 mg/l $Ca(OH)_2$	11.5	39	64	93
Aliquot 2	Chemically Treated			40	67	90
Aliquot 3	Carbon Column Effluent			11	70	92
New Rochelle Pilot Plant						
Aliquot 1	Untreated	$Ca(OH)_2$ Dosage Unknown	Unknown	30	78	92
Aliquot 2	Chemically Treated			30	73	93
Aliquot 3	Carbon Column Effluent			9	74	95

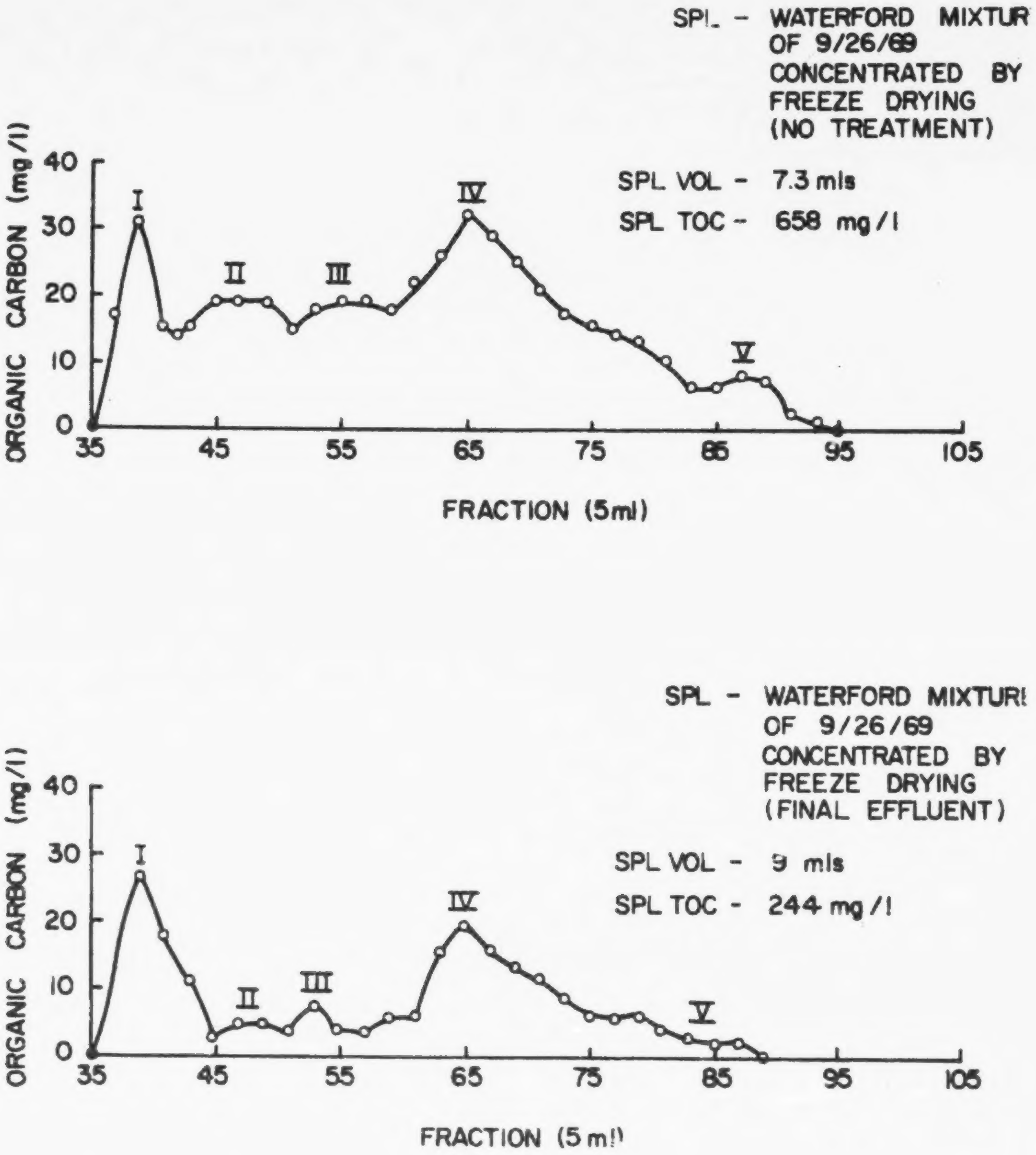

Fig. 11 CHROMATOGRAMS OF INFLUENT AND CHEMICAL-PHYSICAL EFFLUENT - NEW YORK STATE PILOT PLANT SAMPLE OF 9/26/69.

Table 10. Effect of Chemical-Physical Treatment on Waterford Sewage-Paper Waste Mixture of 9/26/69

Apparent Molecular Weight Range	% Recovered Soluble Organic Carbon		Calculated Soluble Organic Carbon in Aliquot (mg/1)		% Soluble Organic Carbon Removal
	Untreated	Column Effluent	Untreated	Column Effluent	
Above 1000	18.7	27.8	7.5	1.95	74
600-1000	14.8	8.4	5.9	0.60	90
100-600	53.4	50.2	21.4	3.52	83
Less than 100	13.2	13.6	5.3	0.95	82

Table 10. Effect of Chemical-Physical Treatment on Waterford Sewage-Paper Waste Mixture of 9/26/69

Apparent Molecular Weight Range	% Recovered Soluble Organic Carbon		Calculated Soluble Organic Carbon in Aliquot (mg/1)		% Soluble Organic Carbon Removal
	Untreated	Column Effluent	Untreated	Column Effluent	
Above 1000	18.7	27.8	7.5	1.95	74
600-1000	14.8	8.4	5.9	0.60	90
100-600	53.4	50.2	21.4	3.52	83
Less than 100	13.2	13.6	5.3	0.95	82

to a slightly lesser degree.

Sample set of 10/10/69. Chromatograms of the concentrated influent, chemical treatment effluent and carbon column effluent for 10/10/69 are shown in Figure 12. The untreated and chemically treated concentrates show five peaks while the carbon column effluent concentrate shows four. It appears that peak II was nearly completely removed. The high lime treatment had no noticeable effects.

Table 11 presents data on removals, percentages and concentrations of soluble organic carbon in various apparent molecular weight ranges. About 87% of the soluble organic carbon in the carbon column effluent is attributed to organics below 1,000 in apparent molecular weight. Removals of soluble organic carbon were high except for the 100 to 600 apparent molecular weight range.

New Rochelle sample set. Chromatograms of the concentrated influent, chemical treatment effluent and carbon column effluent from the New Rochelle pilot plant are shown in Figure 13. The chromatogram for New Rochelle influent sewage concentrate is very similar to previous sewage concentrate chromatograms and indicates a predominance of low apparent molecular weight organics. The chromatographic results for the chemical treatment effluent concentrate show nearly the same characteristics and exhibit a low concentration of apparent high molecular weight organics. The carbon column effluent concentrate again exhibits a predominance of apparent low molecular weight organics. The characteristic peak II (apparent molecular weight 270) found in all other sewage concentrates is absent and is assumed to have been removed by the activated carbon.

4. Analysis of Unconcentrated Samples and Validation of the Freeze-drying Technique

Introduction. The Elnora, New York sewage was used exclusively during this phase. The experimental procedure used is shown in Figure 6. The sample was split into three smaller 1.5 liter samples. The first was not treated, the second was treated with lime to a pH of 11.6 and the third was treated with sodium hydroxide to a pH of 11.6. The untreated and lime treated samples were each further divided into two separate aliquots 3 and 4 (lime treated). Aliquots 2 and 3 were

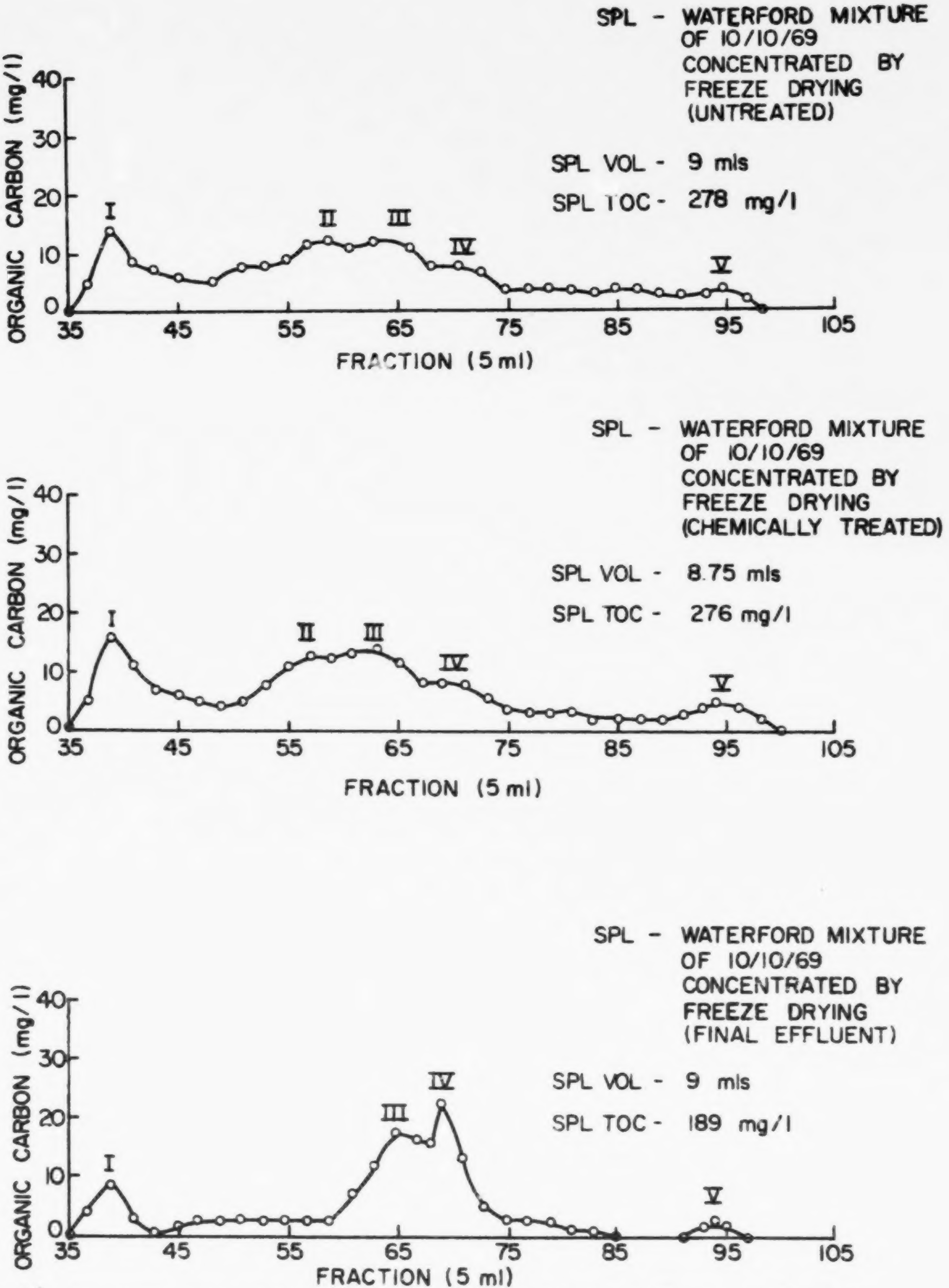

Fig. 12 CHROMATOGRAMS OF CONCENTRATED INFLUENT, CHEMICAL TREATMENT EFFLUENT AND CARBON COLUMN EFFLUENT - NEW YORK STATE PILOT PLANT SAMPLES OF 10/10/69.

Table 11. Effect of Chemical-Physical Treatment on Waterford Sewage-Paper Waste Mixture of 10/10/69

Apparent Molecular Weight Range	% Recovered Soluble Organic Carbon in Range			Calculated Soluble Organic Carbon in Aliquot			% Soluble Organic Carbon Removal
	Untreated	Chemical Effluent	Carbon Column Effluent	Untreated	Chemical Effluent	Carbon Column Effluent	
Above 1,000	16.3	18.2	12.9	6.4	7.3	1.40	78
600-1,000	13.5	12.3	5.8	5.3	4.9	0.64	88
100-600	44.6	47.4	71.5	17.4	19.0	7.85	55
Less than 100	25.5	22.1	9.9	10.0	8.8	1.09	89

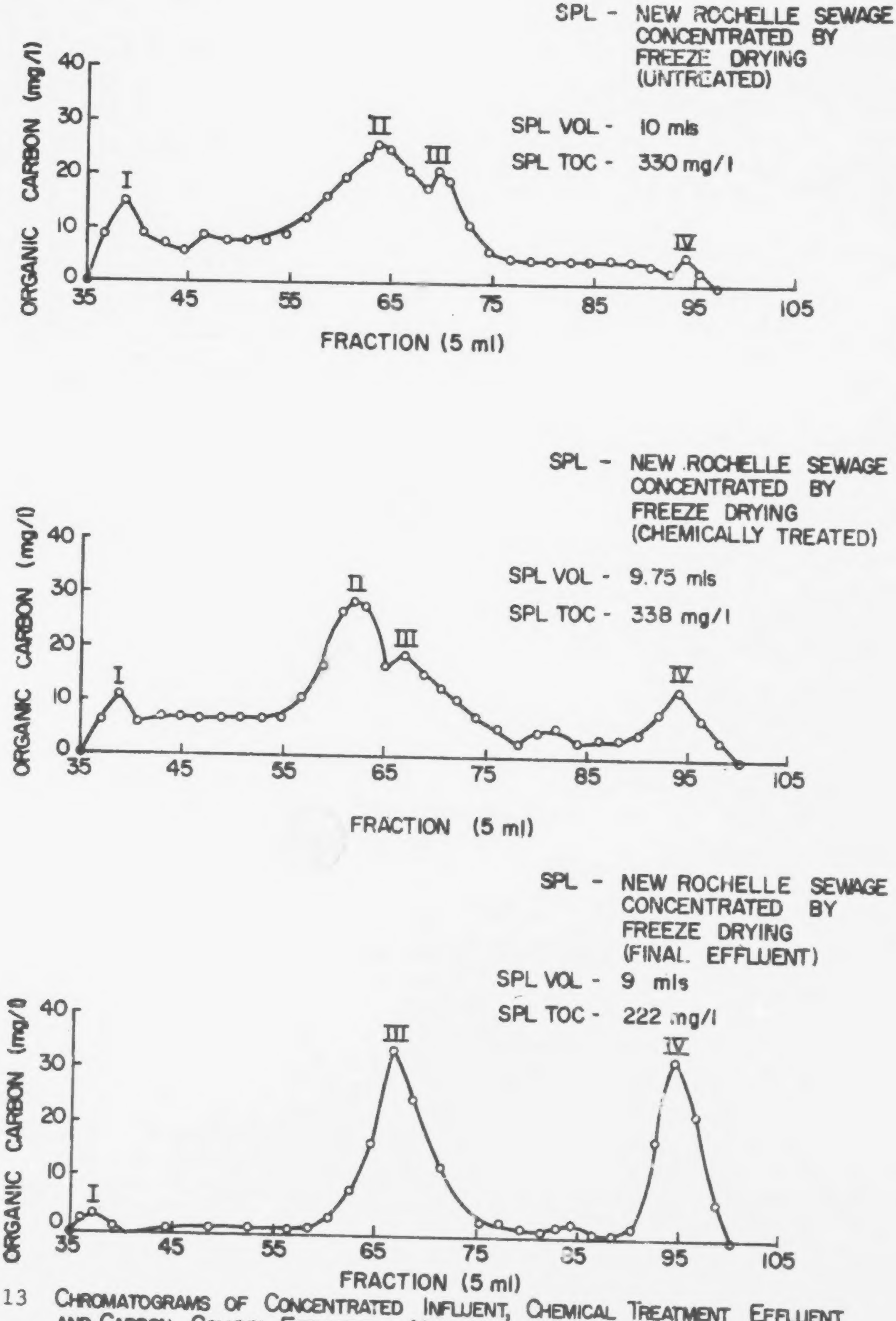

Fig. 13 Chromatograms of Concentrated Influent, Chemical Treatment Effluent and Carbon Column Effluent – New York Rochelle Pilot Plant.

concentrated by freeze-drying. The sample which was treated with sodium hydroxide was used unconcentrated only and is designated as aliquot 5. Ten ml samples of each aliquot were fractionated and analyzed using either the high range (concentrated) or low range (unconcentrated) of the modified carbonaceous analyzer described previously.

The chemical treatment and soluble organic carbon recovery data are presented in Table 12. Lime treatment produced a detectable but not a large drop in soluble organic carbon while sodium hydroxide treatment did not. Lime treatment produced excellent clarification while sodium hydroxide yielded a turbid supernatant.

Validity of the freeze-drying technique. Chromatograms of the concentrated and unconcentrated aliquots of untreated and lime treated sewage are shown in Figures 14 and 15 respectively. Elnora sewage samples. There is little difference between the chromatograms for concentrated and unconcentrated samples.

Table 13 summarizes the percentages of soluble organic carbon in each peak and the amount of carbon each peak represents in the aliquot. Both the percentages and soluble organic carbon concentrations obtained from concentrate analysis agree very closely with the analysis of the unconcentrated aliquots. It is obvious that freeze-drying produced a minimal change in chromatographic results. Peak IV in each concentrate appears to have a slightly lower concentration than its unconcentrated counterpart. The other peaks agree so closely that any difference could be ascribed to experimental accuracy.

Lime versus sodium hydroxide treatment. Figure 16 depicts chromatograms for the unconcentrated aliquots (aliquots 1, 3 and 5). Sodium hydroxide produced practically no effect as evidenced by the similarity between the chromatograms for the untreated and NaOH treated wastewater. Lime appears to have caused a slight decrease in peaks I and IV.

Table 14 summarizes the percentages of recovered soluble organic carbon in each peak and the corresponding organic carbon concentration of the peak in each aliquot. Less than 3% of the soluble organic carbon in the untreated aliquot was in the high apparent molecular weight category (peak I > 1,500). Lime treatment produced a 50% decrease

Table 12. Treatment and Carbon Recovery Data Elnora Sewage

Aliquot	Chemical Dosage	pH Attained	Soluble Organic Carbon (mg/1)		Freeze Dryer Recovery	Column Recovery % Org. C.
			Before Treatment	After Treatment		
1	None	-----	62	62	Unconc.	99%
2	None	-----	62	62	74%	108%
3	321 mg/1 $Ca(OH)_2$	11.6	62	60	Unconc.	95%
4	321 mg/1 $Ca(OH)_2$	11.6	62	60	70%	98%
5	NaOH	11.6	62	62	Unconc.	103%

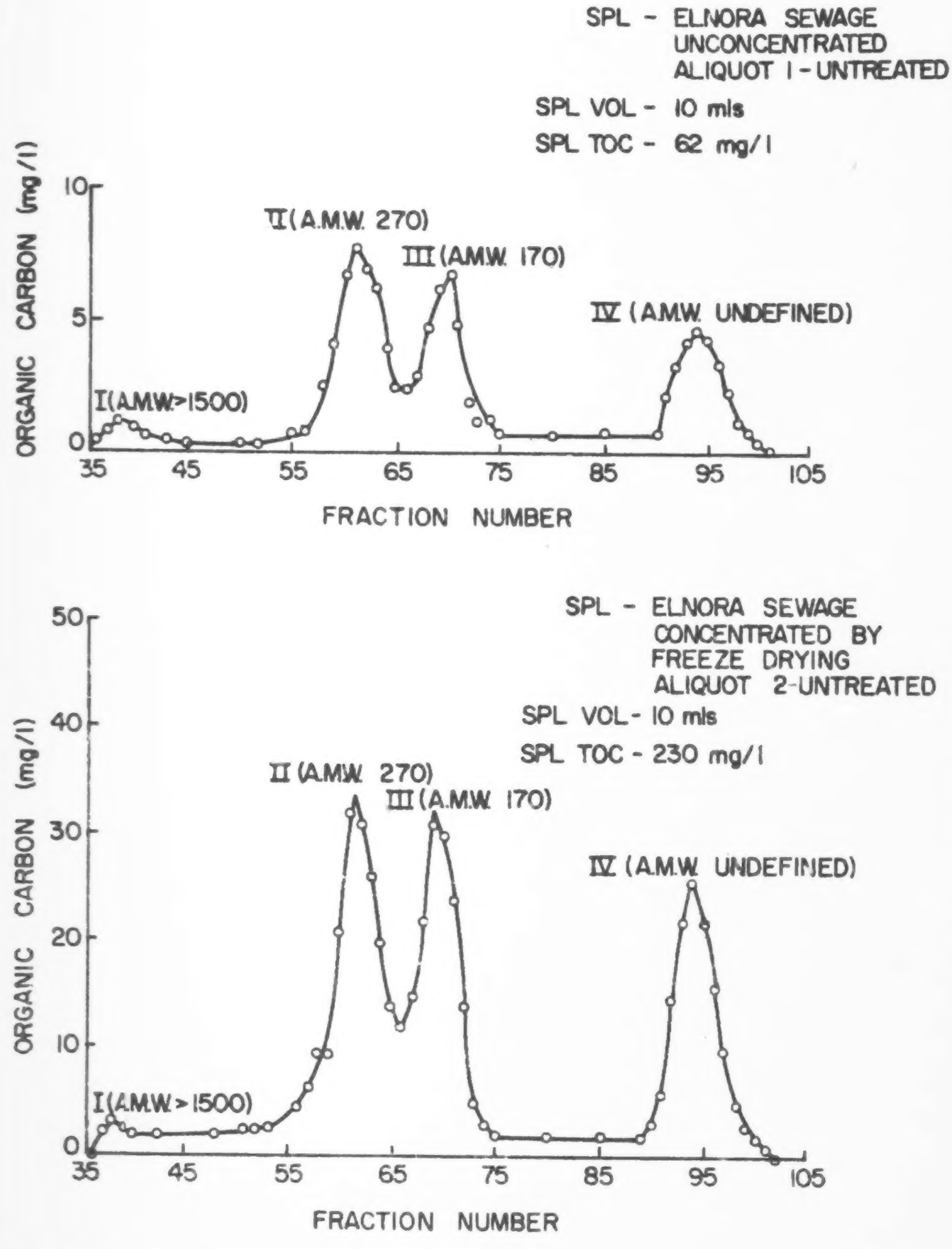

Fig. 14 CHROMATOGRAMS OF UNTREATED AND CONCENTRATED ELNORA SEWAGE.

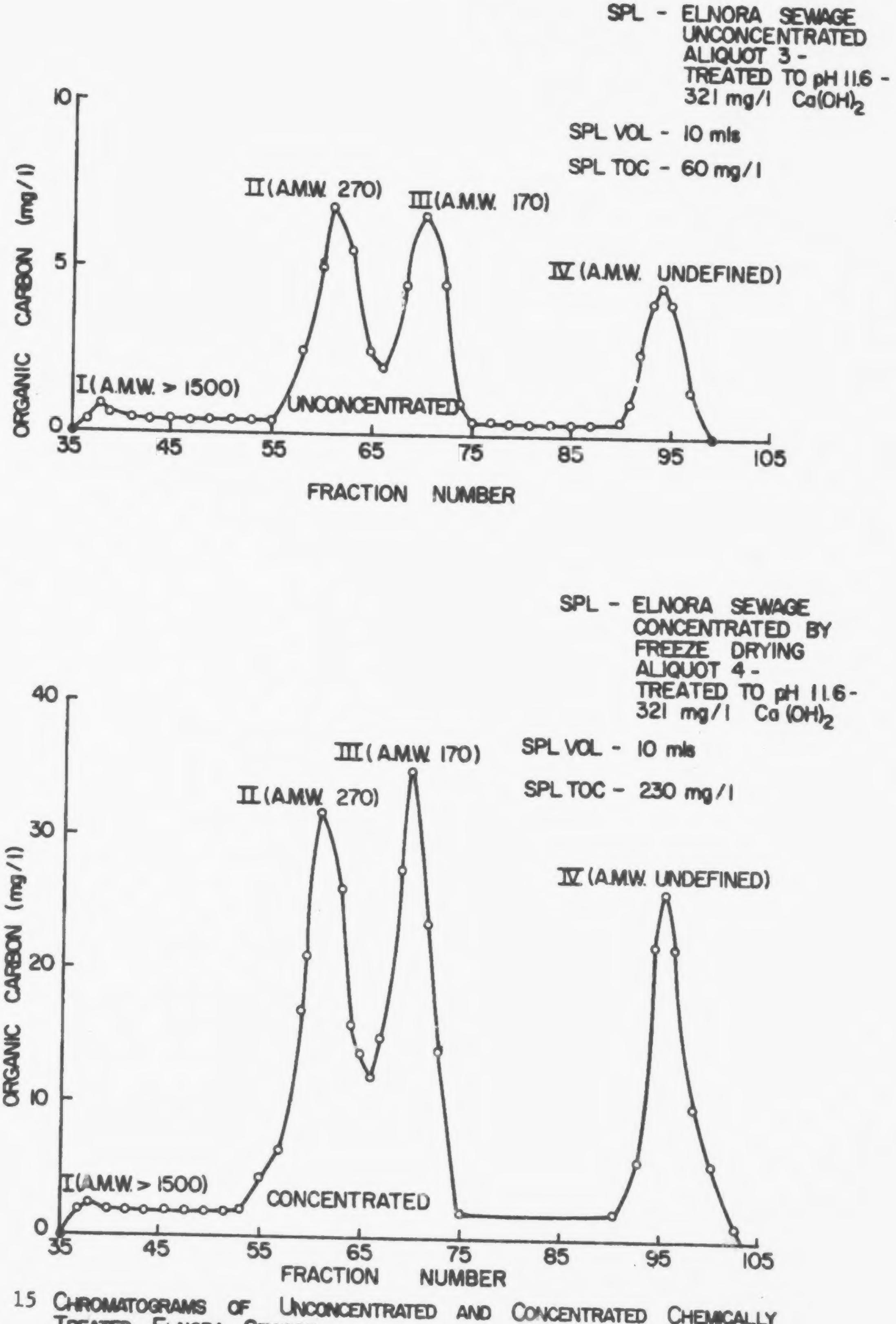

Fig. 15 CHROMATOGRAMS OF UNCONCENTRATED AND CONCENTRATED CHEMICALLY TREATED ELNORA SEWAGE.

Table 13. Effect of Concentration by Freeze Drying on Untreated and Lime Treated Samples of Elnora Sewage

Peak	Apparent Molecular Weight	% Recovered Soluble Organic Carbon in Peak				Calculated Soluble Organic Carbon in Original Sample mg/1			
		Untreated		Lime Treated		Untreated		Lime Treated	
		Unconc.	Conc.	Unconc.	Conc.	Unconc.	Conc.	Unconc.	Conc.
I	1,500	2.8	2.3	1.4	1.4	1.7	1.4	0.8	0.8
II	270	37.8	37.3	37.2	35.5	23.4	23.1	22.3	21.3
III	170	27.2	31.2	28.6	30.0	16.9	19.3	17.2	18.0
IV	Undefined Kav > 1	24.4	18.0	27.7	24.2	15.1	11.2	16.6	14.5

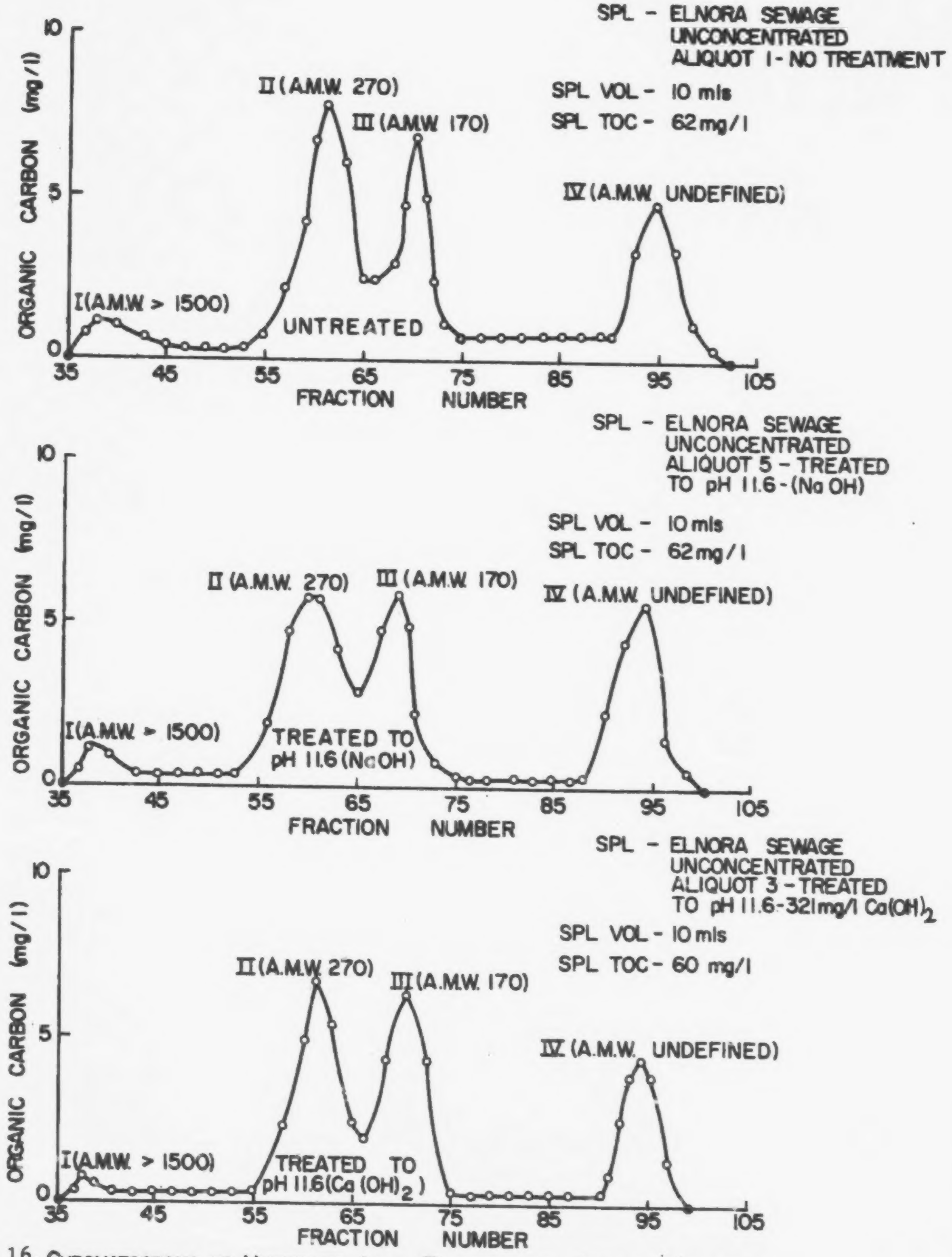

Fig. 16 CHROMATOGRAMS OF UNTREATED, LIME TREATED AND SODIUM HYDROXIDE TREATED ELNORA SEWAGE.

Table 14. Effect of Chemical Treatment on Unconcentrated Elnora Sewage

Peak	Apparent Molecular Weight	% Recovered Soluble Organic Carbon in Peaks			Soluble Organic Carbon in Sample mg/1		
		Untreated	Lime Treated	NaOH Treated	Untreated	Lime Treated	NaOH Treated
I	1,500	2.8	1.4	2.3	1.7	0.8	1.4
II	270	37.8	37.2	39.0	23.4	22.3	24.2
III	170	27.2	28.6	27.3	16.9	17.2	16.9
IV	Undefined Kav > 1	24.4	27.7	26.1	15.1	16.6	16.2

in peak I while sodium hydroxide produced little decrease. These data coupled with the fact that lime treatment caused a drop in soluble organic carbon indicate that a removal mechanism and not pH caused the effect observed.

A summary of these results is presented at the end of the following section (Section VI).

SECTION VI: DISCUSSION

1. Possible Effects of Colloidal Organic Matter on Chromatographic Results

The literature on sewage constituents defines the colloidal fraction as particles between one nannometer and one micron in size (16, 17, 35, 37). Roughly 10% of the organics in sewage can be attributed to the colloidal organic fraction and 40% to the soluble organic fraction (16, 17, 31, 35). The filter used in this work (0.45 micron) was separating in about the middle of the colloidal range; therefore, small amounts of colloidal organics were probably present in the samples analyzed. Based on the swollen size of the gel particles, it can be assumed that at least some sub 0.45 micron colloids would pass through the gel bed. Colloids would be completely excluded from the gel and would appear in the exclusion peak (peak I) as high apparent molecular weight material. In view of the foregoing it is highly probable that an unknown portion of the organics in the exclusion peaks can be attributed to organic colloids.

2. Apparent Molecular Weight Distribution of Domestic Sewage

The fact that all five untreated sewage samples analyzed all exhibited the same four peaks indicates strongly that the nature of the soluble organic fraction is similar between sewages from different locations. This is further confirmed by the fact that two independent studies (17, 31) have resulted in the identification of many of the same soluble organic constituents or organic groups (sugar, organic acids, amino acids, etc) and by considering the origins of domestic sewage. If commercial and industrial wastes are excluded there is little or no variability in the sources that make up sewage (human body wastes, ground garbage, wash water, etc) as evidenced by the fact that such measurements as solid content, BOD content, and many other sewage parameters vary little between sewage sources and in fact have become quite standardized.

This study indicates that high molecular weight substances, as defined by the exclusion limit of Sephadex G-15 of 1,500, comprise less than 10% of the soluble organics in sewage as

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measured by organic carbon. The only study comparable is that reported by Zuckerman and Molof (54, 55) which indicates a much higher proportion of high molecular weight substances. The remaining literature (17, 31) is not conclusive but does report many low molecular weight organic compounds in sewage.

The peak identified as peak IV in all sewage samples could not be defined as to apparent molecular weight since the organics in this peak were obviously under non-steric influences. In all probability this peak contained low molecular weight organics since non-steric influences are reported to increase with decreasing molecular size and increased degree of cross-linking of the gel (4, 11). Sephadex G-15 is a tightly cross-linked gel. One large group that is known to be retarded by Sephadex gels is the aromatic and heterocyclic organic compounds including bile pigments, certain amino acids, indol and skatol (23). Urea is also strongly retarded(23). All of the foregoing organics could be contained in sewage.

The existence of four peaks in a sewage sample indicate that only four soluble organic compounds are predominant. It has been clearly shown that many soluble organic compounds are present in sewage (17, 31). In the case of peaks II, III and IV, each peak represents a group of soluble organics with similar chromatographic characteristics which, due to limitations in resolution of the gel column, appear as one peak theoretically of approximately the same molecular weight. Peak I would theoretically include all compounds with a molecular weight of 1,500 or above (4, 11).

3. Effect of Lime Treatment

High pH lime treatment produced no effect on the high apparent molecular weight material in the Waterford sewage-paper waste mixture (see Figure 12) and in no case did lime treatment cause a dramatic shift in apparent molecular weight distribution as reported by Zuckerman and Molof (54, 55). A consistent but variable reduction in the amounts of high apparent molecular weight organics (peak I) in sewage did occur with lime treatment. The cause of this phenomenon is most probably a removal mechanism such as coagulation, sorption or complexing and not a hydrolysis effect (54, 55).

This is indicated by the data in three ways:

Lime treatment of sewage was accompanied by a drop in soluble organic carbon from the system.

High pH-sodium hydroxide treatment did not produce the degree of reduction in peak I in Elnora sewage as high pH-lime treatment.

A lime dosage sufficient to produce a pH of about 9.5 accomplished very nearly the same effect on peak I in Waterford sewage as a lime dosage sufficient to produce a pH in excess of 11.0. A pH of 9.5 at ambient temperature and pressure is generally considered by biochemists as a rather mild environment for hydrolysis of organics.

4. Apparent Molecular Weight Distribution of Chemical Physical Effluents

All three chemical-physical effluents analyzed contained both high and low apparent molecular weight material; however, the low apparent molecular weight material was predominant. Although organics in some molecular weight ranges were removed more efficiently than others, there was only one case where removals within a range were poor (see Table 11). This predominance of low apparent molecular weight material is supported by data indicating that the soluble organics of the untreated wastes were also of predominantly low apparent molecular weight. Further confirmation is given by other workers (6, 27) who state that low molecular weight hydrophilic organics would be resistant to activated carbon adsorption. The data presented are in substantial disagreement with the reports of Zuckerman and Molof (54, 55) that low molecular weight soluble organics are preferentially absorbed by carbon columns.

5. Effect of Freeze-drying on Chromatographic Results

The fact that freeze-drying did not change the chromatographic characteristics of the organics in untreated and lime-treated Elnora sewage indicates that the nature of the organics was not changed. This is not surprising since freeze-drying has been used effectively for many years in the food and pharmaceutical industries as well as in medicine in handling labile organics (12).

Freeze-dryer recoveries were comparable to those reported by other researches (25, 31) and no large selected losses of organic carbon occurred as indicated by the chromatographic results with Elnora sewage. The losses experienced are attributed to mechanical losses during processing. This is supported by the work of McDonald (25) whose data indicate that about 90% of the loss in organic carbon in freeze-drying wastewater can be accounted for by acid washing the glassware and filters used in preparing concentrates.

6. Summary

The results can be summarized as follows:

Chromatograms of five samples of domestic sewage from four separate locations all exhibited striking similarity showing four characteristic peaks at apparent molecular weights of greater than 1,500, 270, 170 and undefined. The undefined peak appeared after the elution of one gel bed volume and, therefore, has a partition coefficient (Kav) of greater than one, indicating non-steric influences. The vast majority of the soluble organic carbon in sewage is attributed to soluble organics of less than 1,500 in apparent molecular weight.

Dosing sewage with lime did not create any dramatic effects on the chromatographic results. A partial but consistent reduction of the small quantities of the high apparent molecular weight peak (peak I) in sewage was observed. In some cases variable reductions in low apparent molecular weight peaks also were observed. Dosing a mixture of Waterford sewage and paper waste with lime created essentially no effect. In the case of Waterford sewage, a relatively low lime dosage created essentially the same effect as a relatively high dosage. In the case of Elnora sewage, sodium hydroxide produced little effect on peak I while lime produced a reduction in peak I; furthermore, lime produced a decrease in soluble organic carbon during treatment in all cases. This indicates that the effects observed were probably due to a removal mechanism.

Freeze-drying produced little effect on the chromatographic results and all indications are that this technique is an excellent method of concentrating soluble organics in wastewater.

Chromatograms of three final effluents after chemical treatment and activated carbon adsorption indicate that the soluble organics remaining after chemical-physical treatment are primarily of low apparent molecular weight.

Soluble organic carbon removals in all molecular weight ranges were generally high after chemical-physical treatment.

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APPENDIX C

MATERIALS AND APPARATUS

Materials

This section describes the types of permanent equipment and expendable supplies employed in this overall investigation. When appropriate, the specific phase of the study in which the equipment was used is noted parenthetically.

The chemicals used throughout all phases of this experimental work were reagent grade, unless stated otherwise.

The gels used in the experimental work were of the Sephadex type, manufactured by Parmacia Fine Chemicals, Inc., Piscataway, New Jersey. The specific Sephadex gels employed were as follows:

Sephadex G-10. With a particle size range of 40-120 microns and a fractionation range of 0-700 molecular weight for peptides, globular proteins and dextran fractions.

Sephadex G-15. Sephadex G-15, cross-linked, dextran gel was used to form the gel bed. The approximate fractionation range of this gel extends up to 1,500 in molecular weight. Compounds above 1,500 in molecular weight are excluded for the gel.

Sephadex G-25 medium. With a particle size range of 50-150 microns, fractionation range 1,000-5,000 molecular weight for peptides and globular proteins.

Sephadex G-50 medium. With a particle size range of 50-150 microns, fractionation range 1,000-30,000 molecular weight for peptides and globular proteins.

1. Calcium Hydroxide Suspensions (Appendix B)

The calcium hydroxide ($Ca(OH)_2$) suspensions, used in the chemical treatment studies, were made up in 10 g/l, 20 g/l, 30 g/l, and 40 g/l concentrations. These reagents were freshly prepared before use.

2. Sodium Hydroxide Solution (Appendix B)

A 1N sodium hydroxide (NaOH) solution was used in the chemical treatment studies. This reagent was freshly prepared before use.

3. Sulphuric Acid Solutions (Appendix B)

0.05N, 0.10N and 0.50N sulphuric acid (H_2SO4) solutions were used for pH adjustments.

4. Mercuric Chloride Solution (Appendix B)

A 40 g/l mercuric chloride ($HgCl_2$) solution was used to preserve some waste samples (44).

5. Hydrochloric Acid Solution (Appendix B)

A 2N hydrochloric acid (HCl) solution was used in making organic carbon determinations.

6. Oxalic Acid Solutions (Appendix B)

The oxalic acid ($H_2C_2O_4 \cdot 2H_2O$) solutions used to standardize the carbonaceous analyzers were made up to contain 2 mg/l, 4 mg/l, 6 mg/l, 8 mg/l, 10 mg/l, 19 mg/l, 38 mg/l, 57 mg/l, 76 mg/l, 95 mg/l, of organic carbon.

7. Phosphate Buffered Eluent (Appendix B)

The phosphate buffered eluent used in gel permeation chromatography studies was prepared from a stock solution containing 22.57 g/l of KH_2PO_4, 57.74 g/l of K_2HPO_4 and 88.68 g/l of $Na_2\ H\ PO_4$ (54). Buffered eluent was prepared in four liter batches as needed by diluting 40 ml of the stock solution to 4 liters with double-distilled water. The resulting buffer had an ionic strength of 0.0185.

Gel standards (Appendix B) The gel column standards were made up as follows:

Standard No. 1. 1 g/l raffinose ($C_{18}H_{32}O_{16}$, molecular weight 594), 2.5 g/l glicose ($C_6H_{12}O_6$, molecular weight 180), 1.0 g/l egg albumin (molecular weight approximately 10,000). An unknown portion of the egg albumin did not go into solution.

Standard No. 2. 0.5 g/l sucrose ($C_{12}H_{22}O_4$, molecular weight 342).

Standard No. 3. 5.85 g/l sodium chloride (NaCl, molecular weight 58.5).

Standard No. 4. Standard No. 4 consisted of blue dextran 2,000. Blue dextran 2,000 is a high molecular weight carbohydrate (molecular weight approximately 2,000,000) with color groups attached to it to permit visual observation of the compound as it is eluted through the gel column. This standard was made up in sufficient strength to clearly see the sample as it was eluted. The exact concentration of blue dextran in the standard was not determined.

Wastewater samples (Appendix B) Wastewater samples were collected from the following locations and used in laboratory chemical treatment and studies.

Eastern-Western sewage. A four liter grab sample of sewage was collected from the influent sewer to the Eastern-Western sewage treatment plant at about 10 a.m. on June 6, 1969. A second one liter grab sample of this sewage was collected at about 11 a.m. on June 30, 1969. The Eastern-Western sewage treatment plant serves a small residential development of about 50 homes. The sewage is entirely of domestic origin.

Waterford sewage. A 10 liter grab sample of sewage was collected on June 24, 1969 at about 10 a.m. from a manhole located near outfall number 3 of the Waterford, New York sewer system. This system carries both sewage and storm water. There are no known industrial waste discharges tributary to the sewer line sampled.

Elnora, New York sewage. An 18 liter grab sample of sewage was collected from the influent sewer of the Clifton Knolls sewage treatment plant located in Elnora, New York at about 11 a.m. on April 17, 1970. This treatment plant serves a residential development of about 100 homes. The sewage is entirely of domestic origin.

New York State pilot plant. Two separate sets of samples were provided by the New York State Health Department Research Unit for a chemical-physical pilot plant shown and described in Figure 1. A 50% mixture of Mohawk Paper Mill wastes and

PROCEDURE USED:

(1) ADD 15 1/2 GAL. OF 50/50 WATERFORD WASTE TO TANK 1.
(2) ADD CHEMICALS AND RAPID MIX FOR 5 MIN.
(3) FLOCCULATE FOR 15 TO 20 MIN.
(4) SETTLE FOR 2 HOURS
(5) SIPHON SUPERNATENT TO TANK 2 AND MAINTAIN SLOW MIXING.
(6) PUMP SUPERNATENT TO COLUMNS.

OPERATING DATA:

(1) CARBON USED - FILTRASORB 300
(2) COLUMN AREA - 0.0054 SQ. FT.
(3) SURFACE LOADING - 1.5 GPM/SQ.FT.
(4) SUPERFICIAL CONTACT TIME - 60 MIN.
(5) CARBON VOLUME 0.0655 CU. FT.
(6) CARBON WEIGHT - 1.7 LBS.

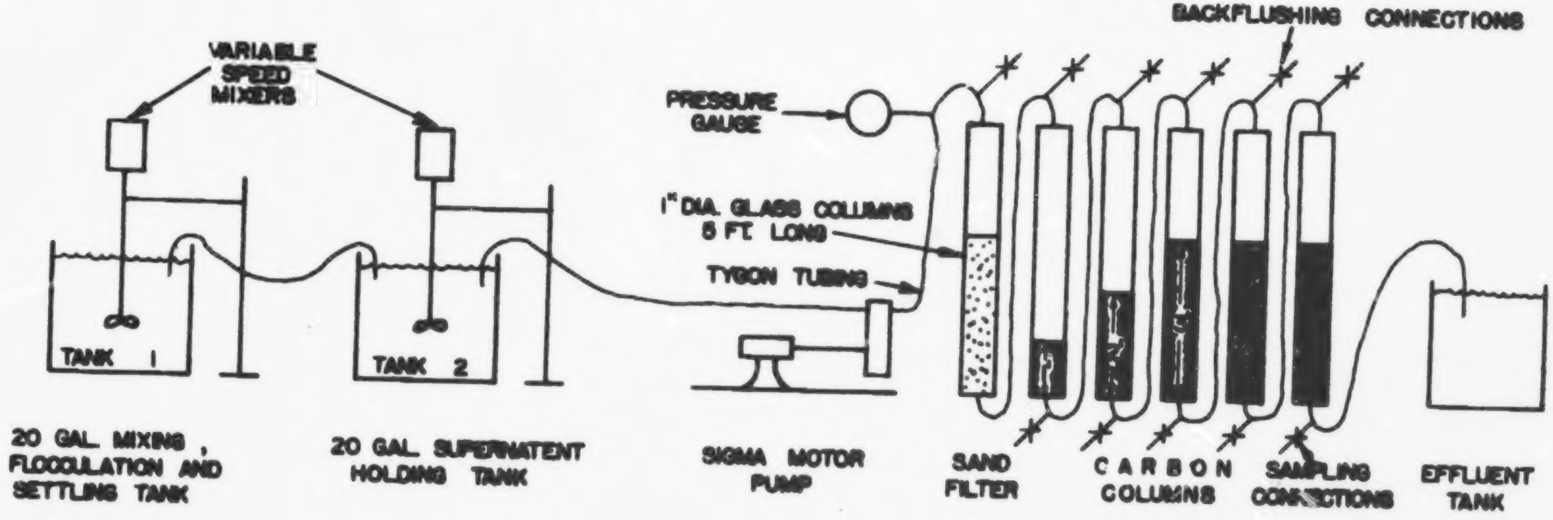

VARIABLE SPEED MIXERS
TANK 1
TANK 2
20 GAL. MIXING, FLOCCULATION AND SETTLING TANK
20 GAL. SUPERNATENT HOLDING TANK
PRESSURE GAUGE
1" DIA. GLASS COLUMNS 5 FT. LONG
TYGON TUBING
SIGMA MOTOR PUMP
BACKFLUSHING CONNECTIONS
SAND FILTER
CARBON COLUMNS
SAMPLING CONNECTIONS
EFFLUENT TANK

Fig. 1 SCHEMATIC OF NEW YORK STATE PILOT PLANT.

Waterford, New York sewage was being treated in daily batches by this plant.

The first sample set consisted of composite samples of the raw waste mixture and final effluent of September 26, 1969. The chemical dosages used on that day were 88.5 mg/l of calcium hydroxide ($Ca(OH)_2$) and 17.7 mg/l of ferric chloride ($FeCl_3$). The pH of the chemically treated waste, before neutralization, was 9.5.

The second set of samples consisted of composite samples of the raw waste mixture, neutralized chemically treated effluent, and final effluent of October 10, 1969. The chemical dosage used on that day was 222 mg/l of calcium hydroxide. The pH of the waste before neutralization was 11.5.

New Rochelle pilot plant. One set of grab samples was provided by the New York State Health Department, Research Unit from the New Rochelle pilot plant which has been described previously (56). The pilot plant was treating the influent waste to the City of New Rochelle, New York sewage treatment plant by the hydrolysis adsorption process which was previously described. New Rochelle is a city of about 75,000 people and the sewage is presumed to contain some commercial and industrial wastes in addition to domestic wastes.

The sample set consisted of raw wastes, chemically treated effluent and final effluent and was collected at about 4 p.m. on October 19, 1969. The samples were not composited in accordance with plant detention time.

Other materials used (Appendix A)

- Bunsen burner
- Drying oven
- Test tubes
- 2,000 ml round bottom flask
- 1.2 liter freeze-drying flask
- Rubber gloves
- Hammer, tongs and safety glasses
- Funnel
- Tygon tubing and pinch clamps
- Plumb bob
- 50 ul syringe

10 ml syringe
Water aspirator
Vacuum hose
Hot plate
Thermometer to read from 0°C to 100°C
Evaporating dish
Membrane filters, 0.45 u pore size. (Millipore)
20 liter plastic carboys
McLeod vacuum gauge

Apparatus

1. Centrifugation and Filtration Equipment

Continuous centrifuge. Model CFR-2, 1,800 ml capacity, 12,000 rpm, manufactured by Lourdes Instrument Corporation.

Filtration apparatus. 147 mm filtration unit used with type HAWP 0.45 u membrane filters. Millipore Corporation, Bedford, Massachusetts.

2. Concentration Equipment

Virtis large port freeze-dryer. Model 10-200, ten liter capacity. The standard model is equipped with eight 3/4" ports and eight 1/2" ports; to increase the rate of sublimation the freeze-dryer was specifically manufactured with sixteen 3/4" ports.

Eight liter dry ice capacity extender. Model 10-137E.

Virtis McLeod gauge. Model 10-224, range 5 microns-5 mm Hg.

Virtis filter seal freeze-drying flasks. 1,200 ml capacity, Model F-128.

All of the above items were manufactured by the Virtis Company, Inc., Gardiner, New York.

Calab thin film rotary evaporator. Model 200, equipped with a 500 cl cold finger condenser and 2,000 ml boiling flask. California Laboratory Equipment Company, Oakland, California.

Welch duo-seal vacuum pump. Model 1402, Skokie, Illinois.

3. Chromatographic Equipment

Sephadex chromatographic columns. 2.5 cm by 100 cm K25/100 fitted with upflow adapters, manufactured by Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.

Fraction collector. Type 3402 B, LKB RadiRac LKB Produkter AB, Stockholm, Sweden.

25 mm syringe filter holders. Equipped with type HAWP 0.45 micron filters, catalogue number SX0002500, Millipore Corporation.

Conductivity bridge. Model 31, manufactured by Yellow Springs Instrument Corporation, Yellow Springs, Ohio.

Microliter syringes. Model 705, 50 microliter capacity, and Model 710, 100 microliter capacity. Hamilton Company, Whittier, California.

pH meter. Corning Model 7 equipped with a temperature compensating electrod.

Total organic carbon analyzer. The laboratory carbonaceous analyzer is designed to measure and record the concentration of small amounts (1 to 100 ppm carbon) of organic material in a sample of water. The major instrumental components include (1) a supply of oxygen, (2) a system for sample introduction and transport, (3) a muffle furnace, (4) infrared analyzer, (5) amplifier and (6) a read out system (recorder).

The new Beckman Model 915 Total Organic Carbon Analyzer used in this work contains two separate but similar channels for the total carbon and inorganic carbon analyses. In the total carbon channel the combustion tube is packed with cobalt oxide impregnated asbestos fiber and is enclosed in a high temperature furnace (950°C). Both the organic and inorganic carbonaceous material is oxidized to CO_2 in the presence of high temperature, oxygen and the catalyst. In the inorganic carbon channel the combustion tube contains quartz chips wetted with 85% phosphoric acid and is heated by a low temperature furnace (150°C). The acid liberates CO_2 and steam from inorganic carbonates. The operating temperature is sufficiently high for the desired reaction, but is substantially below that required to oxidize the organic matter (31).

A condenser is provided after each combustion tube to remove the condensed steam from the effluent which, then, flows to a select valve. This valve is manually operated and is selected for the particular channel in which the sample is injected. The outlet from the select valve is connected to a filter and then to the infrared analyzer which is sensitized to carbon dioxide. A schematic flow diagram for the carbon analyzer showing all the components is presented in Figure 2.

The infrared analyzer, Model 200 LIRA supplied by Mine Safety Appliances, was used to measure the concentration of CO_2 produced when the sample is injected into the Beckman furnace described on the previous page. It consists of two separate infrared sources, a detector, an amplifier and a meter, (Figure 3). Two similar helics of nichrome filaments are heated to 1,200°F by the application of 5.7 volts A.C. The infrared energy beams from these filaments travel through parallel cells. One beam traverses the sample cell; the other beam, the comparison cell. The emergent radiation is directed to a single detector cell. The temperature and pressure of the gas in the detector is accordingly increased by adsorption of radiation (32).

A rotating interrupter alternately blocks the radiation entering the sample cell and the comparison cell. When the gas to be analyzed is introduced into the sample cell, it absorbs some infrared energy and thus reduces the radiation reaching the detector from the sample cell. The two beams become unequal. The detector gas expands and contracts as the beams pass through it from comparison cell and sample cell, respectively. The microphone membrane moves in response to the pressure changes of the detector gas. The condenser microphone capacity varies with the membrane movement, thereby, generating an electrical signal proportional to the difference between the two beams which, in turn, corresponds to the amount of carbon dioxide in the sample cell. This signal is amplified and rectified so that it may be read on the LIRA meter or an external recorder. A Beckman Ten-Inch Laboratory Potentiometric Recorder, Model 1005, was used in this work.

The amplifier is tuned so that only variations in infrared intensity occurring at the interrupting frequency produce an output signal. When no CO_2 is passed through the sample cell the beams are equal and the output is zero. Increased

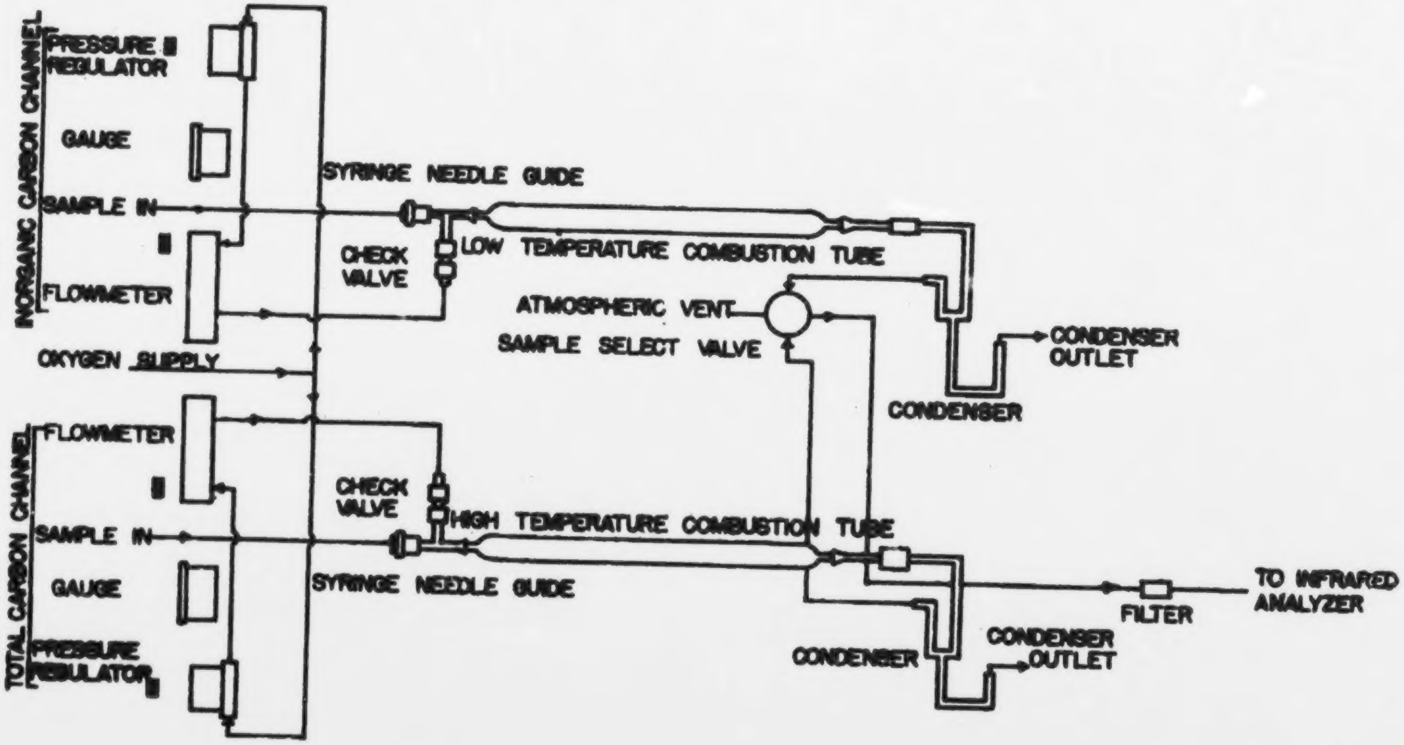

INORGANIC CARBON CHANNEL
PRESSURE REGULATOR
GAUGE
SAMPLE IN
FLOWMETER
OXYGEN SUPPLY
TOTAL CARBON CHANNEL
FLOWMETER
SAMPLE IN
GAUGE
PRESSURE REGULATOR
SYRINGE NEEDLE GUIDE
CHECK VALVE
LOW TEMPERATURE COMBUSTION TUBE
ATMOSPHERIC VENT
SAMPLE SELECT VALVE
CONDENSER
CONDENSER OUTLET
CHECK VALVE
HIGH TEMPERATURE COMBUSTION TUBE
SYRINGE NEEDLE GUIDE
CONDENSER
CONDENSER OUTLET
FILTER
TO INFRARED ANALYZER

Fig. 2 SCHEMATIC DIAGRAM OF TOTAL ORGANIC CARBON ANALYZER

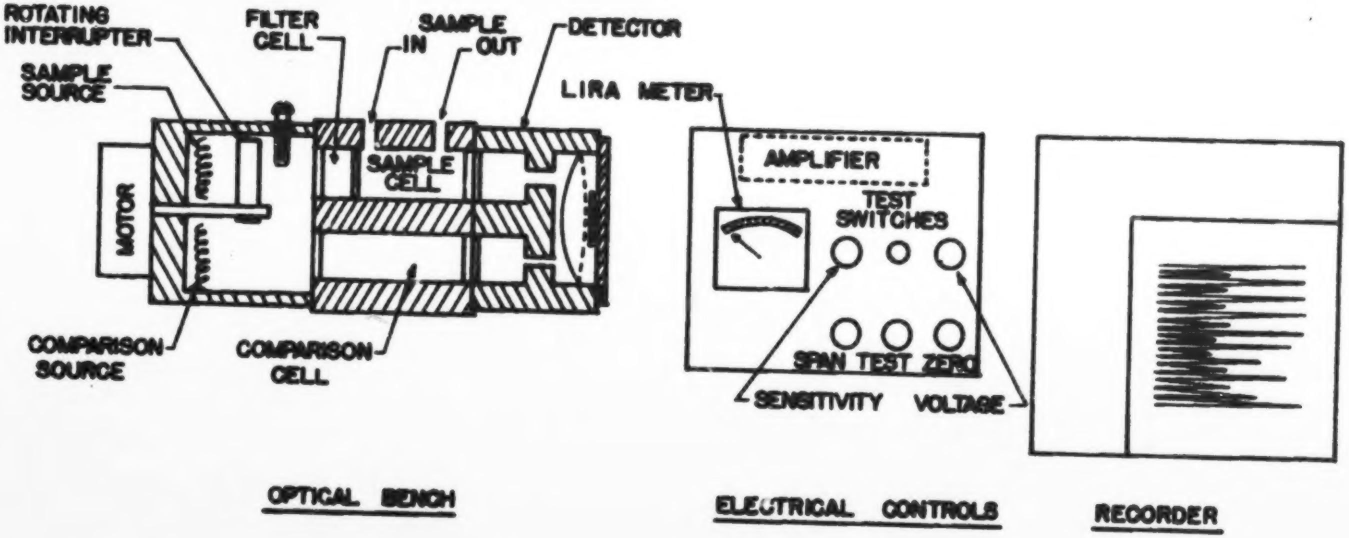

ROTATING INTERRUPTER
SAMPLE SOURCE
FILTER CELL
SAMPLE
IN
OUT
DETECTOR
SAMPLE CELL
MOTOR
COMPARISON SOURCE
COMPARISON CELL
LIRA METER
AMPLIFIER
TEST SWITCHES
SPAN TEST ZERO
SENSITIVITY
VOLTAGE
OPTICAL BENCH
ELECTRICAL CONTROLS
RECORDER

Fig. 3 SCHEMATIC DIAGRAM OF MSA MODEL 200 INFRARED ANALYZER

sample concentration may be simulated by upsetting the optical balance of the analyzer. This is done by operating the sensitivity test switch which increases the comparison beam voltage.

4. Biological Equipment

Microscope. Spencer Company, Buffalo, New York.

Syringes. Model 810L10N Luer-Lok tip disposable syringes, 10 ml capacity. Becton, Dickinson and Company, New York.

Continuously stirred batch culture apparatus. Constructed and depicted on Figure 4.

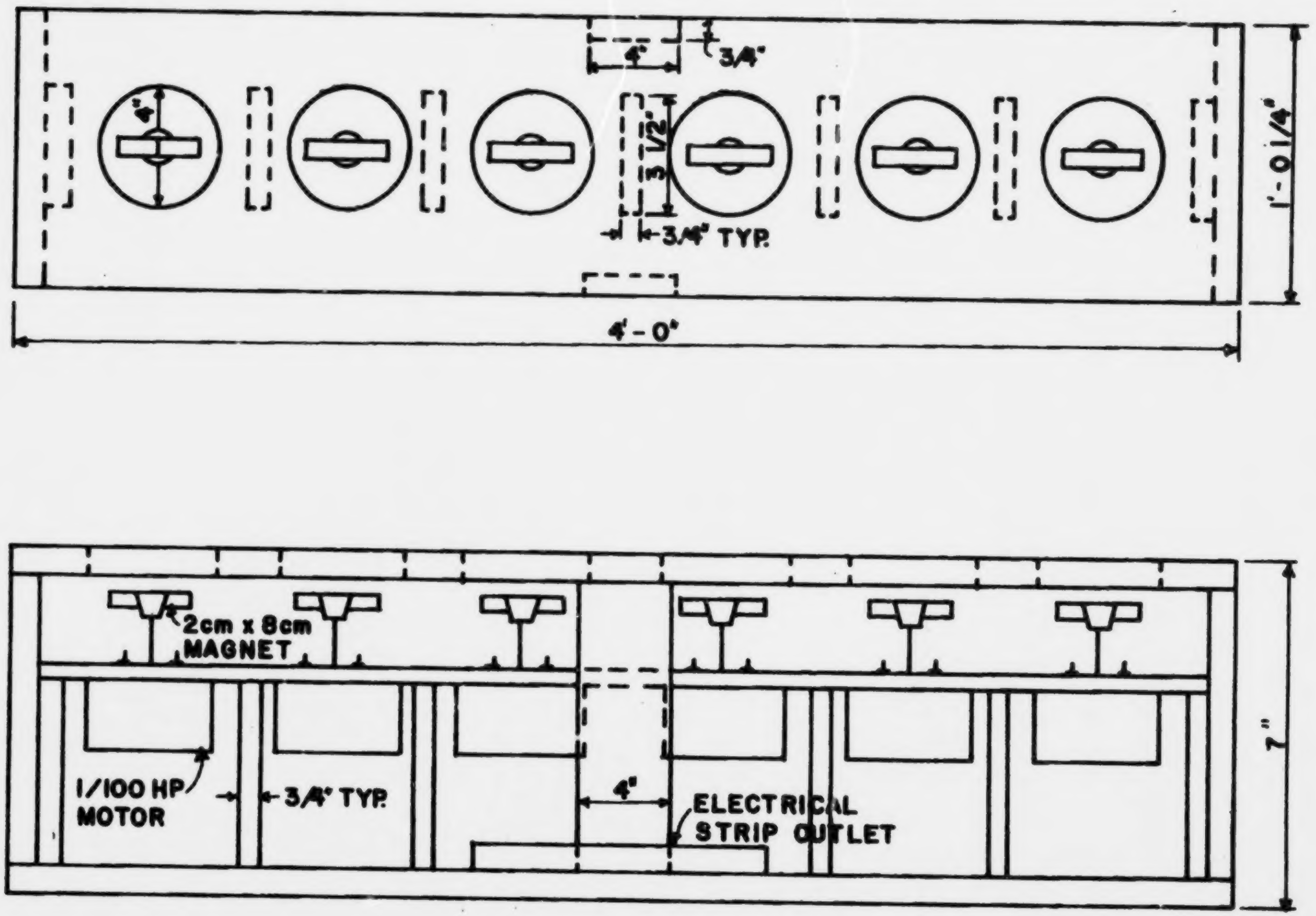
Culture apparatus motors. 1/100 HP, 115V, 1,520 rpm, manufactured by Dayton Electric Manufacturing Company, Chicago, Illinois.

Analytical balance. In the preparation of culture medium, stock solutions and chromatographic standards, a Sartorius 200 g/0, 1 mg Selecta Rapid Balance was used. This balance was also employed in making dry weight determinations of the cultures. Sartorius Werke, Gottinger, Germany.

Cell enumeration. Bright line haemocytometer, American Optical Corporation.

Absorbence measurements. Beckman Model DU-2 spectrophotometer, A 5 cm cell was used in making the absorbence measurements.

Dry weight determinations. 47 mm Hydrosol filter holder and type HAWP 0.45 micron filter, Millipore Corporation.


4"
3/4"
3 1/2"
4"
3/4" TYP.
1'-0 1/4"
4'-0"
2cm x 8cm
MAGNET
1/100 HP
MOTOR
3/4" TYP.
4"
ELECTRICAL
STRIP OUTLET
7"

CULTURE APPARATUS

Fig. 4 CONTINUOUSLY STIRRED BATCH CULTURE APPARATUS.

*U.S. GOVERNMENT PRINTING OFFICE: 1973-546-309/53 I-3